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MÉLATONINE ET L'HYPOXIE-RÉOXYGENATION RÉGULENT DIFFÉREMMENT L'AUTOPHAGIE ET L'INFLAMMATION DANS LE TROPHOBLASTE PLACENTAIRE HUMAIN

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RÉSUMÉ

L'apport en oxygène dans le placenta est finement régulé et directement lié avec le bon déroulement de la grossesse et du développement fœtal. Dans les pathologies de grossesses associées avec un remodelage artériel incomplet, telles que la prééclampsie, l'apport d'oxygène est plus faible et intermittent entraînant une hypoxie/réoxygénation (H/R). Cette H/R est associée avec une augmentation du stress oxydatif et de l'apoptose des trophoblastes villeux. Il n'existe aucun traitement thérapeutique ou préventif pour la prééclampsie excepté l'accouchement. La mélatonine est produite *de novo* par les cellules trophoblastiques humaines. Cette indolamine exerce diverses actions protectrices telles que antioxydante, anti-inflammatoire et régulation de l'autophagie. Au niveau placentaire, la mélatonine protège les trophoblastes des dommages induits par l'H/R, entre autres, par une action antioxydante directe et indirecte, via ses récepteurs MT1 et MT2. Par contre, sa capacité de diminuer l'inflammation et de réguler l'autophagie induite par une H/R trophoblastique n'a jamais été étudiée. Les hypothèses de recherche sont : la mélatonine (1) via la régulation de l'autophagie et de l'inflammation protège le trophoblaste villeux contre les dommages induits par l'H/R ; et (2) module différemment l'autophagie pour maintenir la survie/l'homéostasie des cellules trophoblastiques normales et induire la mort des cellules tumorales. L'objectif général est de comprendre comment la mélatonine module l'autophagie des cellules placentaires (primaires et tumorales) en situation de stress. Les objectifs spécifiques sont 1) Déterminer si et par quelle voie de signalisation la mélatonine régule l'inflammation et l'autophagie dans le syncytiotrophoblaste primaire humain cultivé en normoxie et H/R ; 2) Déterminer si la mélatonine régule différemment l'autophagie dans les cellules trophoblastiques normales (primocultures) et tumorales (cellules BeWo). Les expériences ont été réalisées avec la lignée cellulaire BeWo (lignée cellulaire de choriocarcinome placentaire humain) et avec des cultures primaires de trophoblastes villeux de placentas normaux à terme. Toutes les expériences ont été conduites sous conditions de normoxie (8 % O_2) mimant l'espace intervilleux. Pour mimer l'H/R, la normoxie a été suivie par 4 h d'hypoxie (0,5 % O_2) puis 18 h de normoxie. Les cellules ont été traitées ou non avec 1 mM de mélatonine à toutes les 24 h. L'expression protéique des biomarqueurs de l'autophagie, de l'inflammation et de l'apoptose a été déterminée par immunobuvardage de type western, par immunofluorescence, par immunohistochimie et à l'aide de la trousse MILLIPLEX MAP ; la mélatonine a été mesurée par ELISA. L'exposition *in vitro* du syncytiotrophoblaste à l'H/R réduit la production endogène de mélatonine et induit une réponse pro-inflammatoire par l'augmentation des facteurs TNF, IL-6 et NFκB. Au contraire, le traitement avec 1 mM mélatonine augmente les taux d'IL-10 et rétablit les taux de TNF, d'IL-6 et NFκB aux taux observés en normoxie. L'H/R augmente également l'autophagie dans le syncytiotrophoblaste. La mélatonine et l'inhibition spécifique du NFKB induisent également l'autophagie et augmente la survie cellulaire en condition d'H/R. Dans les cellules BeWo, l'H/R avec et sans mélatonine réduit la viabilité cellulaire et induit l'activation des capteurs d'énergie cellulaire comme le AMPK et le PP2A-C, qui sont des modulateurs en amont de l'autophagie. Dans les BeWo, l'H/R a induit la formation de l'autophagosome, l'activité autophagique et l'activité du facteur de transcription Nrf2. La mélatonine, dans les conditions d'H/R, a réduit l'activation de l'autophagie et du Nrf2, ce qui a entrainé l'activation de l'apoptose. Dans les primocultures de cytotrophoblastes villeux, l'H/R a aussi induit l'autophagie et Nrf2. Par contre, la mélatonine a augmenté l'activité de l'autophagie et du Nrf2 protégeant les cellules primaires contre les effets de l'H/R. Puisque la mélatonine est impliquée dans les mécanismes de survie cellulaire des trophoblastes primaires, ses actions pourraient avoir un effet bénéfique sur la santé de la grossesse et sur le développement du fœtus.

Mots clés: Mélatonine; Autophagie; Inflammation; Placenta; Trophoblaste villeux: Reproduction

ABSTRACT

The oxygen supply in the placenta is finely regulated and directly related to the good progress of pregnancy and fetal development. In addition, in pregnancy pathologies associated with incomplete arterial remodeling, such as pre-eclampsia, the oxygen supply is lower and intermittent resulting in hypoxia/reoxygenation (H/R). The H/R is at the origin of the trophoblastic oxidative stress and trophoblastic and maternal inflammation. H/R is associated with increased oxidative stress and apoptosis of villous trophoblasts. There is no therapeutic or preventive treatment for these pathologies that can reduce their effects before delivery. In addition, melatonin is produced *de novo* by human trophoblast cells. Melatonin exerts various protective actions such as antioxidants, anti-inflammatory and via the regulation of autophagy in various tissues. At the placental level, melatonin protects trophoblasts from H/R-induced damage, including direct and indirect antioxidant action through its MT1 and MT2 receptors. On the other hand, its ability to decrease H/R-induced trophoblastic inflammation and to regulate the trophoblastic autophagy has never been studied. The research hypotheses are: melatonin (1), via the regulation of autophagy and inflammation, protects the villous trophoblast against H/R-induced damages; and (2) modulates autophagy differently to maintain normal trophoblastic cell survival/homoeostasis and induce tumour cell death. The overall goal is to understand how melatonin modulates placental (primary and tumour) cell autophagy in stressful situations. The specific objectives are 1) to determine if and by which signalling pathway melatonin regulates inflammation and autophagy in the human primary syncytiotrophoblast cultured in normoxia and H/R; 2) determine if melatonin regulates autophagy differently in normal trophoblastic cells (primary cell culture) and tumours (BeWo cells). The experiments were carried out on the BeWo cell line (human placental choriocarcinoma cell line), on primary cultures of villous trophoblasts of normal placentas at term. All experiments were conducted under normoxic conditions (8% O_2) mimicking the intervillous space. To mimic an H/R situation, normoxia was followed by 4 h of hypoxia (0.5% O_2) and then 18 h of normoxia. The cells were treated or not with 1 mm melatonin every 24 hours. The protein expression of the biomarkers of autophagy, inflammation and, apoptosis was determined by western immunoblotting, immunofluorescence and immunohistochemistry. The quantification of inflammatory factors was performed using the MILLIPLEX MAP KIT with magnetic beads; melatonin was measured by ELISA. In vitro exposure of the syncytiotrophoblast to H/R reduces the endogenous production of melatonin and induces a pro-inflammatory response by increased TNF, IL-6 and NFκB factors. In contrast, melatonin treatment increases IL-10 levels and restores TNF, IL-6 and NFκB levels. H/R also increases autophagy. Melatonin and a specific inhibitor of NFκB also lead to activation of autophagy and increased cell survival under H/R conditions. In BeWo cells, H/R with and without melatonin reduces cell viability and induces the activation of cellular energy sensors, which are upstream modulators of autophagy. H/R induces the autophagosome formation, autophagic activity, and induces Nrf2 transcription factor activity. In H/R conditions, melatonin reduced the activation of both autophagy and Nrf2, leading to the activation of apoptosis. In normal placental cells, H/R induces the activation of autophagy and Nrf2. Melatonin also increases their activity and consequently protects the cells against the deleterious effects of H/R. Since melatonin is involved in the regulation protective mechanisms of the human placenta, the actions of melatonin could improve the trophoblastic protection and sustain an optimal pregnancy and fetal development. Further studies are needed to optimize the effects of melatonin as possible prophylaxis or treatment for pregnancy diseases, such as preeclampsia.

Keywords: Melatonin; Autophagy; Inflammation; Placenta; Villous Trophoblast; Reproduction

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- AANAT: Arylalkylamine N-acetyltransférase
- AMK : *N^1-acetyl-5-methoxykynuramine*
- AMPc : Adénosine monophosphate cyclique
- ATG : *Autophagy-related*
- ATP : Adénosine triphosphate
- Bax : *bcl-2-like protein 4*
- Bcl-2 : *B-cell lymphoma 2*
- Bid : *BH3 interacting-domain death agonist*
- BNIP3 : *BCL2/adenovirus E1B 19 kDa protein-interacting protein 3*
- Class III PI3K : *Phosphatidylinositol 3-kinase*
- CMA : *chaperone-mediated autophagy*
- COX-2 : cyclooxygénase
- DAPK : *Death associated protein kinase*
- DISC : *Death-inducing signalling complex*
- ERO : Espèce oxygénée réactive
- evCTB : Cytotrophoblastes extravilleux
- hCG : Hormone gonadotrope chorionique humaine
- HIF: Hypoxia-inducible factor
- HLA : Antigène des leucocytes humain
- hPL : Hormone lactogène placentaire
- H/R : Hypoxie/réoxygénation
- IFN-γ : Interféron gamma
- IL : Interleukine
- iNOS : Oxyde nitrique synthase inducible
- IP3 : Inositol 1,4,5 triphosphate
- IRE1 : Inositol-requiring enzyme 1
- JNK : *c-Jun N-terminal kinase*
- LC3B : *Light chain 3*
- NFκB : *Nuclear factor kappa B*
- NQO2 : quinone reductase 2
- PI3K : *Phosphatidylinositol-4,5-bisphosphate 3-kinase*
- PLA2 : Phospholipase A2
- PLC : Phospholipase C
- RCPG : Récepteurs couplés aux protéines G
- RCIU : Restriction de croissance intra-utérine
- ROR : *Retinoid orphan receptor*
- RZR : *Retinoid Z receptor*
- SA : Artère spiralée utérine
- SMAC : *Second mitochondrial activator of caspases*
- sENG : Endogline soluble
- STB : Syncytiotrophoblaste
- Th1 : Lymphocytes T de type 1
- Th2 : Lymphocytes T de type 2

- TNF : Facteur de nécrose tumorale
- TOR : *Target of rapamycine*
- TRAIL: *Récepteurs se liant au ligand induisant l'apoptose*
- Treg : Cellules régulatrices T
- vCTB : Cytotrophoblastes villeux
- XIAP : *X-linked inhibitor of apoptosis protein*

1. ÉTAT DES CONNAISSANCES

1.1. Placenta humain

Malgré son caractère temporaire, le placenta tient un rôle central dans le développement fœtal. Il permet les échanges entre la mère et le fœtus. Il possède des rôles trophiques, endocrines et immunitaires pour garantir le développement fœtal. Le placenta est originaire d'une interaction extrêmement complexe entre le blastocyste et l'endomètre maternel. Le placenta humain est de type hémomonochorial, caractéristique des primates supérieurs. Ce type de placenta permet le contact direct entre le sang maternel et les trophoblastes et, avec les grands primates, est un des uniques capables de produire l'hormone gonadotrope chorionique humaine (hCG) (Cole, 2010, Crosley *et al.*, 2013, Malassine *et al.*, 2003), compliquant l'utilisation de modèle animal.

1.1.1. Développement placentaire

Le zygote, généré par la fusion entre un spermatozoïde et un ovule, subit plusieurs divisions cellulaires avant de prendre le nom de blastocyste. Ce dernier est constitué de deux types cellulaires distincts : les embryoblastes, aussi appelées masse cellulaire interne, localisés au niveau de la face interne du blastocyste, et les trophoblastes, qui forment la couche externe. Ces derniers sont à l'origine de la formation du placenta (Budhraja *et al.*, 2006, Cartwright *et al.*, 2010, Diedrich *et al.*, 2007, Tuuli *et al.*, 2011).

Plusieurs facteurs libérés par l'endomètre et par les trophoblastes sont essentiels à l'implantation du blastocyste dans l'utérus. Parmi ces facteurs, la hCG, synthétisée par le corps jaune au début de la grossesse et par la suite par les trophoblastes joue un rôle majeur notamment dans l'implantation (Garrido-Gomez *et al.*, 2010, Huppertz, 2007, Vicovac *et al.*, 1995). Après l'adhésion du blastocyste à l'endomètre, les trophoblastes commencent à se différencier selon deux voies : la voie des cytotrophoblastes villeux (vCTBs) et la voie des cytotrophoblastes extravilleux (evCTB) (Figure 1). Durant le premier trimestre de la grossesse, les trophoblastes se différencient majoritairement vers la voie des trophoblastes extravilleux ce qui permet l'invasion du blastocyste dans l'utérus et la mise en place de la circulation utéro-placentaire. Après le premier trimestre, la voie des vCTBs deviendra largement majoritaire,

situation qui démontre la capacité adaptative du placenta au cours de la grossesse (Graham *et al.*, 1992a, Ji *et al.*, 2013, Kadyrov *et al.*, 2003).

Les vCTBs, cellules mononuclées, se différencient en syncytiotrophoblaste (STB), cellule multinuclée non-prolifération. Le STB constitue la couche externe de l'arbre villositaire en contact direct avec le sang maternel (Figure 1). Cette localisation privilégiée permet au STB d'avoir un rôle clé dans les échanges mère-fœtus. La surface d'échange entre le sang maternel et le placenta est augmentée par la présence de microvillosités sur la membrane apicale du STB. L'absence de l'antigène des leucocytes humains (HLA) de classe I au niveau du STB permet une bonne tolérance immunitaire, ce qui est très important dut au fait de son contact avec le sang maternel. Un autre rôle important du STB est sa production des hormones protéiques et stéroïdiennes essentielles au bon déroulement de la grossesse (Graham *et al.*, 1992b, Kaufmann *et al.*, 2003, O. Nakamura, 2009, Teasdale *et al.*, 1985).

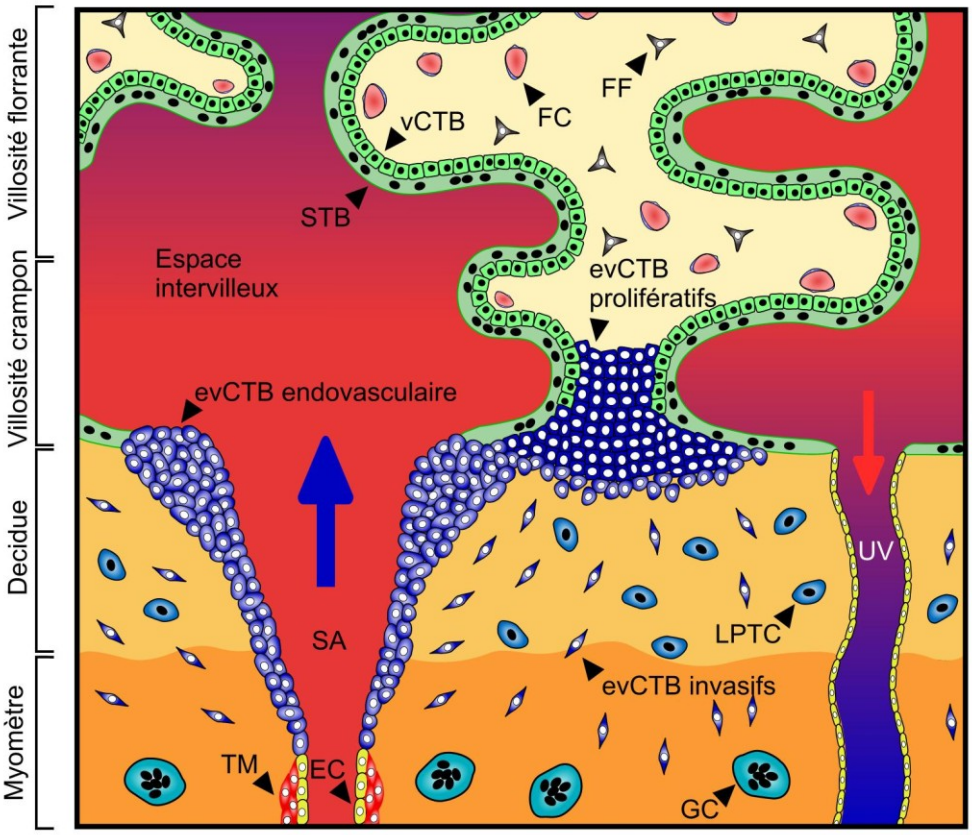

Figure 1: Schéma des composants de l'arbre villositaire. Le sang maternel accède l'espace intervillositaire et effectue les échanges materno-fœtaux avec le syncytiotrophoblaste (STB). vCTB: cytotrophoblastes villeux; evCTB: cytotrophoblastes extravilleux; EC: cellule endothéliale; FC: capillaire fœtal; FF: fibroblaste fœtal; GC: cellules géantes; LPTC: grande cellule polygonale trophoblastique; SA: artère spiralée utérine; TM: tunique musculaire; UV: veine utérine. Modifiée de (Lanoix D. *et al.*, 2012).

La prolifération des vCTBs permet la formation et le maintien des villosités placentaires. Ces villosités, appelées villosités chorioniques, sont formées par deux régions : les villosités crampon ancrées dans le tissu utérin et les villosités flottantes situées dans l'espace intervilleux. La totalité de la superficie des villosités chorioniques est entourée par le STB (Figure 1). L'ancrage de ces villosités dans la décide maternelle est permise grâce aux evCTBs. Les evCTBs se détachent et migrent profondément dans l'utérus. Ils sont constitués de deux types majeurs de cellules: les evCTBs prolifératifs et les evCTBs invasifs (Figure 2) (Handschuh *et al.*, 2009, Handschuh *et al.*, 2007, Ji *et al.*, 2013).

La circulation entre la mère et le fœtus est établie à la fin du premier trimestre de la grossesse et le remodelage des artères spiralées maternelles est terminé à la fin du deuxième trimestre. Les evCTBs invasifs sont responsables de l'invasion des artères, et remplacent les cellules endothéliales par les evCTBs endovasculaires (Figure 1). Ce phénomène modifie les caractéristiques vasculaires, transformant les petites artères musculaires de haute résistance en de gros vaisseaux fibreux de faible résistance, ce qui facilite l'entrée du sang dans les lacunes trophoblastiques (Cartwright *et al.*, 2010, Ji *et al.*, 2013, Kadyrov *et al.*, 2003, Kaufmann *et al.*, 2003).

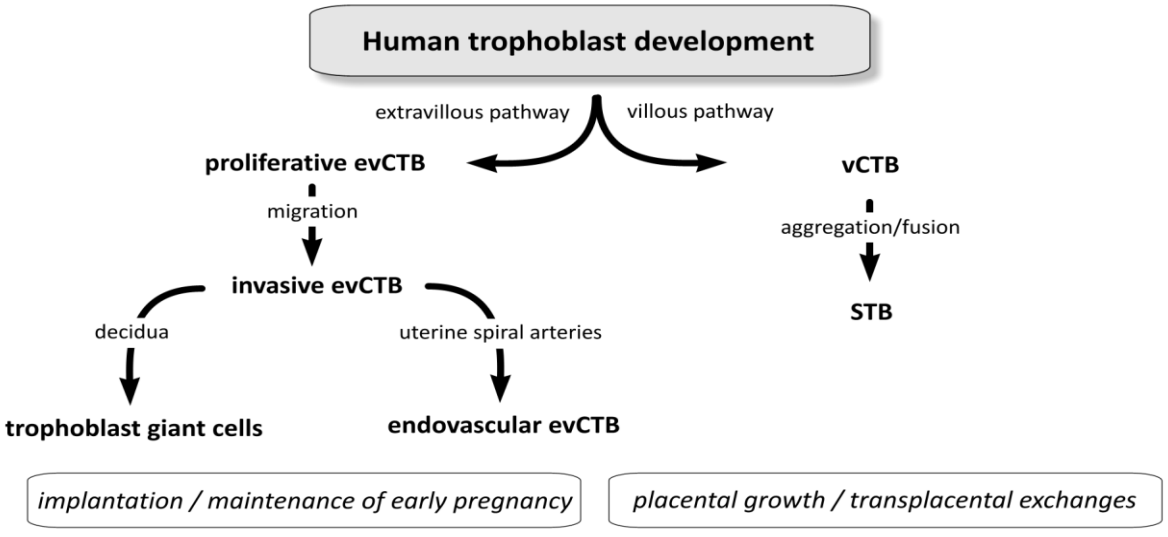

Figure 2 : Voies de différenciation du trophoblaste humain. Après l'adhésion du blastocyste à l'endomètre, les trophoblastes commencent à se différencier selon deux voies : la voie des cytotrophoblastes villeux (vCTBs) et la voie des cytotrophoblastes extravilleux (evCTB). Le phénotype de trophoblaste présent dans les villosités flottantes est le trophoblaste villeux alors que le trophoblaste extravilleux est localisé dans les villosités STB : syncytiotrophoblaste. Tirée de (Vaillancourt *et al.*, 2009a).

1.1.2. Homéostasie du trophoblaste villeux

Le STB est une cellule non proliférative. La différenciation et la fusion des vCTBs avec le STB adjacent doit donc d'être constante et bien régulée pour maintenir l'homéostasie du STB(Mounier *et al.*, 2009, Vaillancourt *et al.*, 2009a). Il est intéressant de constater que les vCTBs ne fusionnent qu'en quantité nécessaire avec le STB et que les vCTBs ne fusionnent pas entre eux *in vivo (Kar et al., 2007, Longtine et al., 2012)*. Ce processus de renouvellement est complexe et implique une grande quantité de facteurs comme des hormones, des facteurs de croissance, des cytokines ou encore des facteurs de transcription (Tableau 1) (Crocker *et al.*, 2007, Kar *et al.*, 2007, Pidoux *et al.*, 2012).

In vitro, le sérum fœtal bovin est un facteur clé pour la différenciation morphologique et biochimique complète des vCTBs en STB (Bischof *et al.*, 2005, Morrish *et al.*, 1987). De façon générale, la différenciation des vCTBs en STB a lieu après 48 h à 72 h de culture. Après 96 h, on observe une diminution de la survie ainsi qu'une apoptose des cellules. Comme le montre la Figure 3, il s'agit d'un processus physiologique observé in vivo pendant toute la grossesse afin de maintenir l'efficacité optimale du placenta. (Crocker *et al.*, 2001). Bien que les deux voies de l'apoptose (intrinsèque et extrinsèque) soient impliquées dans ce processus, la voie intrinsèque est majoritairement retrouvée. Elle est notamment engagée lors des variations de concentrations d'oxygène, de l'hypoxie et du stress oxydatif au niveau du placenta (Crocker *et al.*, 2001, Kar *et al.*, 2007, Lanoix *et al.*, 2013, Longtine *et al.*, 2012).

Tableau 1: Principaux facteurs régulant la fusion des cytotrophoblastes villeux et la formation du syncytiotrophoblaste.

Facteur	Description		Type cellulaire	Action	Références
EGF	Facteur de croissance	+	TT	Biochimique	(Morrish *et al.*, 1987)
CSF	Facteur de croissance	+	TT	Biochimique	(Garcia-Lloret *et al.*, 1994)
VEGF	Facteur de croissance	+	PT, TT	Biochimique	(Crocker *et al.*, 2001)
hCG	Hormone	+	TT	Biochimique	(Shi *et al.*, 1993)
TGF-α	Facteur de croissance	+	TT	Biochimique	(Yang *et al.*, 2003)
TNF	Cytokine	-	TT	Biochimique	(Leisser *et al.*, 2006)
ERK1/2	Protéine kinase	+	TT	Biochimique fusion	(Vaillancourt *et al.*, 2009b)
p38	Protéine kinase	+	TT	Biochimique Fusion	(Vaillancourt *et al.*, 2009b)
PKA	Protéine kinase	+	B	Fusion	(Knerr *et al.*, 2005)
Syncytin 1	Protéine de membrane	+	TT, B	Fusion	(Frendo *et al.*, 2003)
CD98	Transporteur d'acides aminés	+	B	Fusion	(Kudo *et al.*, 2003)
Galectin 3	Protéine de membrane	+	B	Fusion	(Dalton *et al.*, 2007)
Connexin 43	Protéine de membrane	+	TT	Fusion	(Frendo *et al.*, 2003)
Hypoxie	Tension réduite d'oxygène	-	TT, B	Biochimique Fusion	(Alsat *et al.*, 1996)
Calcium	Ion	+	TT, B	Fusion	(Alsat *et al.*, 1996)

(-) : inhibition ; (+) activation ; B : cellules BeWo ; CSF : *Colony stimulating factor* ; EGF : *Epidermal growth factor* ; ERK1/2 : *Extracellular signal–regulated kinases ;* hCG : gonadotrope chorionique humaine ; PKA *:* Protein kinase A ; PT : Premier Trimestre ; TGF-α : *Transforming growth factor alpha* ; TNF : Facteur de nécrose tumorale ; TT : Troisième trimestre ; VSGF : *Vascular endothelial growth factor.*

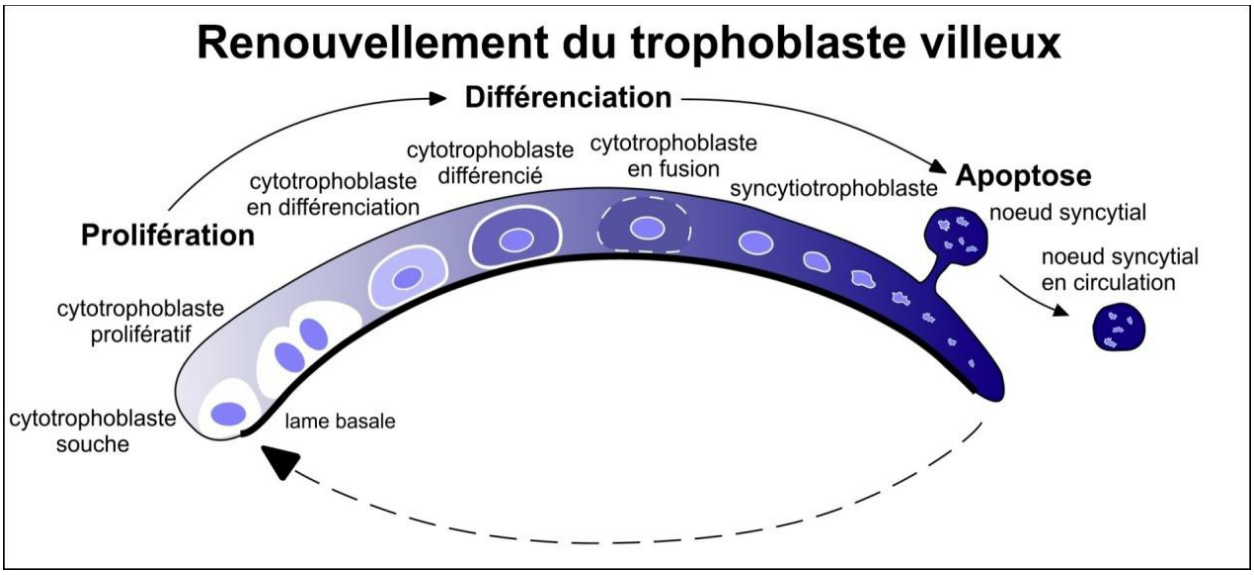

Figure 3: Renouvellement du trophoblaste villeux. Le trophoblaste villeux est formé des cytotrophoblastes souches prolifératifs qui vont sortir du cycle cellulaire et se différentier en cytotrophoblastes villeux. Les cytotrophoblastes villeux mononucléés vont fusionner et se différencier en un syncytium, le syncytiotrophoblaste. La dégénérescence du syncytiotrophoblaste se produit par apoptose pour permettre sa régénération et donc de maintenir l'homéostasie du trophoblaste villeux. Modifiée de (Lanoix D. *et al.*, 2012).

1.1.3. Oxygénation du placenta

Le placenta est un organe qui subit des variations importantes d'oxygénation au cours de la grossesse. Au premier trimestre, le taux d'oxygénation est physiologiquement très bas (2 % O_2) et augmente à un taux de 8 % O_2 au cours du deuxième et troisième trimestre de la grossesse. La réplication *in vitro* de l'environnement *in vivo* des trophoblastes à l'intérieur des villosités placentaires est un défi, car entre autres, les variations des taux d'oxygénation peuvent induire des changements phénotypiques (Jauniaux *et al.*, 2001, Rodesch *et al.*, 1992). Comme décrit dans l'article de revue de Huppertz et al., au cours du premier trimestre il existe des preuves qu'un faible taux d'oxygène aide au maintien des evCTBs dans un phénotype prolifératif et non invasif, via la régulation du facteur *hypoxia-inducible factor* (HIF)-1α (Caniggia *et al.*, 2000, Huppertz *et al.*, 2009). Lorsque l'oxygénation atteint un taux plus élevé les evCTBs changent de phénotype, c'est-à-dire : arrêtent leur prolifération et deviennent invasive. Par

conséquent, ils envahissent le myomètre et atteignent les artères spiralées (Kaufmann *et al.*, 2003, Kemp *et al.*, 2002).

Chen et al. ont étudié de manière approfondie plusieurs variables liées à la tension d'oxygène dans les primocultures de cellules trophoblastiques et démontré l'importance de déterminer le taux d'oxygène dans l'environnement péricellulaire (B. Chen *et al.*, 2013). Le taux d'oxygène dans les villosités placentaires tend à augmenter en raison de la vasculogenèse. Le flux sanguin dans les villosités augmente constamment et le taux de peroxyde d'hydrogène (une espèce oxygénée réactive (ERO) abondante) est un signal qui contrôle la vasculogenèse (Tuuli *et al.*, 2011). Dans les complications de la grossesse, un manque de vasculogenèse génère une hypoxie, et plus important encore, des variations intermittentes de l'oxygénation (appelée hypoxie/réoxygénation (H/R)). Cette H/R entraîne une augmentation anormale du stress oxydatif, qui compromet la viabilité du placenta et du fœtus (Ji *et al.*, 2013, Lanoix *et al.*, 2013, Lanoix D. *et al.*, 2012).

1.1.4. Inflammation du placenta

Les lymphocytes T de type Th1 et Th2 proviennent de cellules T indifférenciées qui se différencie sous l'influence de l'interféron gamma (IFN-γ) et de l'interleukine (IL)-4, respectivement. Les Th1 produisent une série de cytokines inflammatoires, dont l'IFN-γ, l'IL-2 et le facteur de nécrose tumorale (TNF) et sont les principaux effecteurs de la médiation des actions pro-inflammatoires contre les pathogènes intracellulaires (H. J. Lee *et al.*, 2000, Miyatake *et al.*, 2000, O'Garra *et al.*, 2000). Les Th2 produisent l'IL-4, IL-10 et IL-6. Alors que l'IL-4 et l'IL-10 sont considérées comme des cytokines anti-inflammatoires, l'IL-6 a des propriétés anti- et pro-inflammatoires (Sykes *et al.*, 2012).

La définition de la grossesse en tant qu'état anti-inflammatoire a été acceptée avec enthousiasme et de nombreuses études ont tenté de prouver et de soutenir cette hypothèse. Cette théorie postule que la grossesse est une condition anti-inflammatoire et qu'un changement dans le type de cytokines vers un état pro-inflammatoire conduirait à un avortement spontané ou à des complications de la grossesse. Alors que de nombreuses études ont montré des évidences pour cette hypothèse (Szekeres-Bartho *et al.*, 1996, Wegmann *et al.*, 1993), un nombre similaire d'études ont démontré des données contre ce postulat (Granot *et al.*, 2012, Saito *et al.*, 2010). Dans plusieurs études, la grossesse a été évaluée comme ayant un évènement immunologique unique, alors qu'en réalité, elle comporte trois phases immunologiques distinctes qui sont caractérisées par des processus biologiques distincts(Mor

et al., 2017, Mor *et al.*, 2011, Sykes *et al.*, 2012). Au premier trimestre, le blastocyste doit percer la muqueuse épithéliale de l'utérus pour s'implanter (Aplin, 1991, Makrigiannakis *et al.*, 2004, Makrigiannakis *et al.*, 2001). Les evCTBs invasifs remplacent les cellules endothéliales et le muscle lisse vasculaire des vaisseaux sanguins maternels afin d'assurer un apport adéquat de sang fœto-placentaire. Un environnement inflammatoire est nécessaire pour assurer la réparation adéquate de l'endothélium utérin et l'élimination des débris cellulaires (Tarrade *et al.*, 2001). Pendant ce temps, la physiologie de la mère est modifiée pour s'adapter à la présence du fœtus (en plus des changements hormonaux et autres facteurs, cette réponse inflammatoire est responsable des "nausées matinales"). Ainsi, le premier trimestre de la grossesse est une phase pro-inflammatoire (Mor *et al.*, 2011, Red-Horse *et al.*, 2004). La deuxième phase immunologique de la grossesse comprend le deuxième trimestre et une partie du troisième trimestre. Cette phase est une période de croissance et de développement fœtal. La mère, le placenta et le fœtus sont symbiotiques et la caractéristique immunologique prédominante est l'induction d'un état anti-inflammatoire. Les nausées sont alors diminuées, en partie, parce que la réponse immunitaire prédominante est anti-inflammatoire (Makris *et al.*, 2006). La parturition est caractérisée par un afflux de cellules immunitaires dans le myomètre afin d'induire un processus inflammatoire. Cet environnement pro-inflammatoire favorise la contraction de l'utérus, l'expulsion du bébé et la délivrance du placenta (Richter *et al.*, 2009). En conclusion, la grossesse est une condition pro-inflammatoire et anti-inflammatoire, selon le moment de la grossesse (Figure 4).

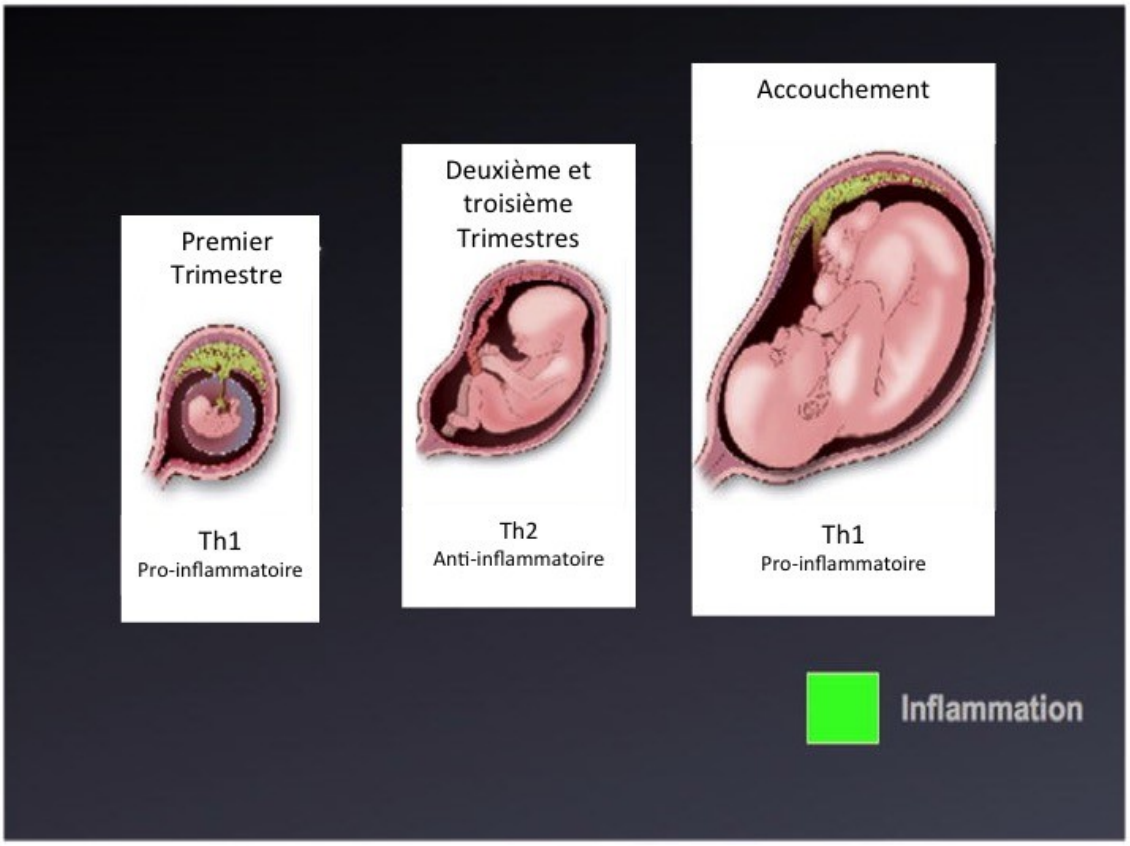

Figure 4: Stades immunologiques de la grossesse. Chaque stade de la grossesse est caractérisé par un environnement inflammatoire unique. Le premier trimestre et l'accouchement sont pro-inflammatoires (Th1), alors que le deuxième et le troisième trimestre représentent une phase anti-inflammatoire (Th2) (Mor *et al.*, 2017, Mor *et al.*, 2011).

Des études indiquent que les cytokines placentaires et maternelles jouent un rôle central chez les patientes atteintes de maladies de la grossesse. Par exemple, dans les accouchements prématurés observés chez les femmes avec une infection intra-amniotique, les concentrations sérologiques et amniotiques de TNF sont plus élevées par rapport à la concentration observée chez les femmes sans infection (Romero *et al.*, 1989). L'administration de TNF et d'IL-1β (pro-inflammatoire) induit un accouchement prématuré chez les primates non humains(Sadowsky *et al.*, 2006). L'IL-10, la principale cytokine anti-inflammatoire des Th2, est fortement diminuée dans le placenta et dans le sérum maternel dans les maladies de la grossesse, comme la prééclampsie et la restriction de croissance intra-utérine (Lai *et al.*, 2011).

La relation entre les cellules immunitaires maternelles et placentaires est intimement liée au privilège immunitaire placentaire. Ce mécanisme demeure mal compris. Cependant, il est connu

pour déterminer le destin de la grossesse. Le privilège immunitaire observé dans les grossesses normales n'est pas seulement lié aux cellules placentaires ; il dépend également du type de cellules T maternelles prédominantes. Comme précédemment décrit le maintien de l'équilibre fin de l'immunité Th1/Th2 est nécessaire pour la grossesse (Figueiredo *et al.*, 2016, Saito *et al.*, 2010). D'autres sous-types de cellules T maternelles sont aussi modulés au cours de la grossesse et donc nécessaires pour une grossesse en santé. Les lymphocytes Th-17 (qui produisent sélectivement IL-17) sont associés à la pathogenèse des maladies auto-immunes et au rejet des structures d'allogreffe (comme l'unité fœto-placentaire) (Figueiredo *et al.*, 2016, Kleczynska *et al.*, 2011). Différentes études ont trouvé des données contradictoires concernant les niveaux de Th-17 dans l'utérus des femmes présentant une grossesse normale (Nakashima *et al.*, 2010, Saito *et al.*, 2010, Santner-Nanan *et al.*, 2009). Dans les cas d'avortement spontané répété, les taux sérologiques et utérins de Th-17, qui induisent l'infiltration tissulaire et l'expression des cytokines pro-inflammatoires, sont considérablement augmentés par rapport aux cas d'interruption de grossesse thérapeutique volontaire (W. J. Wang *et al.*, 2010). Les cellules régulatrices T (Treg) jouent également un rôle dans le contrôle fin de la tolérance immunitaire au cours de la grossesse. Ce sous-ensemble de lymphocytes T est responsable du contrôle de la réponse immunitaire excessive par l'induction de l'expression de cytokines anti-inflammatoires telles qu'IL-10 et TGF- β (Jutel *et al.*, 2003, Samstein *et al.*, 2012). Dans les grossesses normales, le ratio Th-17/Treg est diminué du à une augmentation des taux de Treg (Figueiredo *et al.*, 2016). En revanche, dans les cas de prééclampsie, le ratio Th-17/Treg est altéré avec une augmentation des taux de Th-17 et d'IL-17 et des taux Treg non modifiés dans l'utérus (Vargas-Rojas *et al.*, 2016, W. J. Wang *et al.*, 2010).

1.1.5. Mort cellulaire du trophoblaste villeux

Le processus de fusion est lié aux « stades initiateurs » de la cascade apoptotique dans les vCTBs, alors que l'extrusion des corps apoptotiques du STB est le résultat des « étapes d'exécution finales » de la cascade apoptotique syncytiale. En examinant les profils d'activation des caspases suite à différent stimuli qui induisant l'apoptose, au moins deux voies apoptotiques distinctes ont été identifiées : 1) la voie intrinsèque; et 2) la voie extrinsèque (Huppertz *et al.*, 2006, Walczak, 2013).

Les signaux de mort provenant d'un stress cellulaire, y compris les variations d'oxygène, activent la voie apoptotique intrinsèque (appelé aussi apoptose mitochondriale). La libération dans le cytoplasme du cytochrome c de la mitochondrie induit la formation de l'apoptosome,

favorise l'activation catalytique de la caspase-3 et conduit à la mort cellulaire (Cindrova-Davies *et al.*, 2007, Fischer *et al.*, 2005, Heazell *et al.*, 2008). Contrairement à la voie intrinsèque, la stimulation des récepteurs de mort cellulaire active une apoptose extrinsèque qui ne nécessite pas une implication mitochondriale. Les récepteurs de mort forment un sous-groupe de la superfamille des récepteurs du TNF qui comprend le TNF-R1, le CD95 et les récepteurs se liant au ligand induisant l'apoptose (TRAIL). La machinerie apoptotique de ces récepteurs est activée par ses ligands spécifiques, le TNF, le CD95-ligand (CD95L aussi appelé Fas-L) et le TRAIL. Une fois activé, le récepteur de mort se lie à une des deux pro-caspases apicales (pro-caspase-8 ou pro-caspase-10) (Wachmann *et al.*, 2010) et initie la formation du complexe *death-inducing signalling complex* (DISC) (B. Chen *et al.*, 2010a, Hung *et al.*, 2010). À son tour, la caspase-8 ou caspase-10 induit le clivage du facteur *BH3 interacting-domain death agonist* (Bid), qui se transloque à la membrane extracellulaire des mitochondries pour initier la perméabilisation de la membrane et la libération subséquente des facteurs apoptotiques intermembranaires (Tait *et al.*, 2013, Walczak, 2013). Dans les cellules de type I, la caspase-8 peut induire l'apoptose via le clivage direct de la caspase-3, indépendamment des facteurs pro-apoptotiques mitochondriales. En revanche, les cellules de type II expriment des niveaux plus élevés du *X-linked inhibitor of apoptosis protein* (XIAP) par rapport aux cellules de type I XIAP se lie à la caspase-3 et empêche son clivage direct par les caspases apicales ainsi, dans les cellules de type 2, l'apoptose est donc induite par la voie mitochondriale. La perméabilisation des membranes des mitochondries induit la libération dans le cytoplasme du *second mitochondrial activator of caspases* (SMAC), inhibant l'interaction de XIAP avec la caspase-3 et ainsi le clivage de la caspase-3. L'activation de la caspase-3 entraine la fragmentation et la dégradation de l'ADN ainsi que la condensation de la chromatine. Les dommages à l'ADN vont à leur tour déclencher l'activation du facteur de transcription p53, le principal régulateur de la survie de la cellule, causant l'apoptose. Compte tenu des niveaux élevés de XIAP dans les trophoblastes, l'apoptose trophoblastique est principalement dépendante des mitochondries (Arroyo *et al.*, 2014, Jeon *et al.*, 2013, Paulsen *et al.*, 2011, Paulsen *et al.*, 2008) (Figure 5).

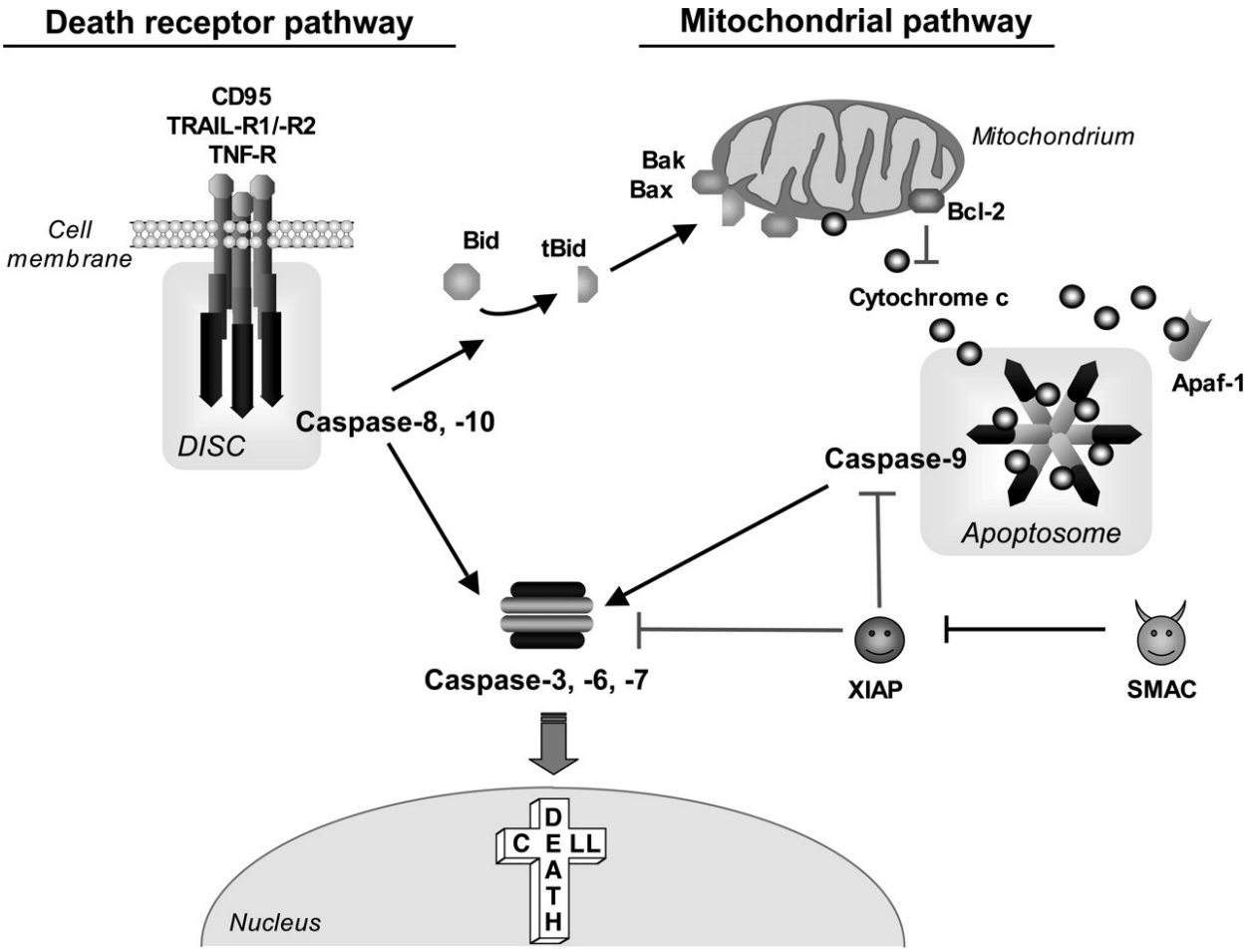

Figure 5: Transduction du signal de l'apoptose. Deux voies majeures de l'apoptose existent dans les trophoblastes placentaires. À gauche, la voie extrinsèque de mort cellulaire contrôlée par un membre de la superfamille des récepteurs du TNF (facteur de nécrose tumorale). La mort cellulaire par le récepteur de mort cellulaire est initiée par le recrutement de protéines adaptatrices spécifiques à chaque récepteur qui se lient à la caspase-8 ou -10. La formation du complexe DISC (*Death-inducing signalling complex*) entraine l'activation de la caspase-8, qui clive et active directement les caspases-3, -6 ou -7, et conséquemment la mort cellulaire des cellules de type I. À droite, dans la voie mitochondriale ou intrinsèque, qui est initiée par de multiples formes de stress, les membres de la famille Bcl-2 (B-cell lymphoma 2) pro-apoptotiques Bax (*Bcl-2-associated X protein*) et Bak (Bcl-2 homologous antagonist/killer) sont transloqués aux mitochondries. La protéine Bid active Bax et Bak pour favoriser la libération du cytochrome c dans le cytosol. Ceci déclenche l'assemblage de l'apoptosome et l'activation subséquente de la caspase-3 et la mort cellulaire. La molécule XIAP se lie directement aux caspases et inhibe leur activité enzymatique. Dans les cellules de type II, la fonction inhibitrice des XIAP (*X-linked inhibitor of apoptosis protein)* est contrée par le SMAC (*Second mitochondrial activator of caspases)*, c'est-à-dire que l'apoptose dépend des facteurs mitochondriaux (tirée de Fischer and Schulze-Osthoff 2005) (Fischer *et al.*, 2005, Kay *et al.*, 2007, Tjoa *et al.*, 2006)**.**

Le CD95 est un récepteur membranaire qui appartient à la famille des récepteurs du TNF. CD95 induit l'apoptose (Hata *et al.*, 1996) et l'inflammation (Legembre *et al.*, 2004), ainsi que la prolifération (Reinehr *et al.*, 2008), la migration (Jarad *et al.*, 2002) et l'invasion (Kleber *et al.*, 2008) cellulaire. Le large spectre des actions de CD95 dépend de son activation par son

ligand CD95L transmembranaire ou CD95L soluble (sCD95L) (Fouque *et al.*, 2014, Jost *et al.*, 2009). Les cellules possédant le CD95L transmembranaire (appelé ici CD95L) sont capables d'induire l'apoptose dans les cellules exprimant CD95. Le sCD95L contribue à la sévérité de l'inflammation et de l'auto-immunité par l'activation du NFκB (Guegan *et al.*, 2017). Tous les trophoblastes placentaires possèdent à la fois CD95 et son ligand. Dans les grossesses normales, l'apoptose du STB est finement régulée pour maintenir l'homéostasie placentaire. Par contre, dans les maladies de grossesse associées à une H/R placentaire l'homéostasie trophoblastique est altérée et associée avec une augmentation de l'apoptose et de la nécrose du STB (Paulsen *et al.*, 2011, Strand *et al.*, 2004, Zorzi *et al.*, 1998).

1.2. Autophagie

Pendant la grossesse, l'inflammation et l'apoptose du trophoblaste placentaire sont activées pour soutenir le développement optimal du fœtus. De même, l'autophagie est un processus activé pour soutenir l'homéostasie trophoblastique. L'autophagie est un mécanisme qui permet à la cellule de cataboliser une partie de son contenu, que ce soit des protéines ou des organites cellulaires, en molécules simples comme les acides aminés, les monosaccarides et les acides gras pour rétablir l'homéostasie et assurer la survie cellulaire. C'est la seule voie qui peut dégrader massivement des macromolécules et des organites, c'est une voie de dégradation alternative à celle du protéasome (Azad *et al.*, 2009, Barth *et al.*, 2010, Debnath *et al.*, 2005). En situations où les taux d'ATP sont réduits, comme en état d'H/R et de photodommage causés par la radiation ultraviolette, l'autophagie est activée (B. Chen *et al.*, 2012a, Coto-Montes *et al.*, 2012). L"autophagie" peut être définie en trois voies : 1) la microautophagie, 2) l'autophagie induite par les protéines chaperonnes (CMA, *chaperone-mediated autophagy*) et 3) la macroautophagie. La microautophagie est une voie active dans les situations où l'ATP est réduite, où les protéines endommagées sont directement absorbées par le lysosome. La CMA est caractérisée par le transport des protéines endommagées directement au lysosome par des protéines chaperonnes. Finalement, la macroautophagie (aussi appelée autophagie) est caractérisée par la formation de l'autophagosome, une structure en double membrane qui transporte les protéines et les organites au lysosome et est la voie autophagique la plus étudiée dans la plupart des organismes (Klionsky *et al.*, 2016).

L'autophagie peut être activé par deux voies distinctes : la voie dépendante de la kinase *mammalian target of rapamycine (m*TOR, aussi appelé *mechanistic target of rapamycin* ou

FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1)) et la voie indépendante de cette kinase. Il existe deux complexes mTOR, mTOR1 et mTOR2 qui régulent des processus cellulaires différents (Eisenberg-Lerner *et al.*, 2009). mTOR1 (sérine / thréonine kinase) régule la croissance, la prolifération, la survie, la motilité cellulaire ainsi que l'autophagie. mTOR2 (tyrosine kinase) favorise l'activation des récepteurs de l'insuline, le facteur de croissance analogue à l'insuline 1 (IGF-1) et les récepteurs de contrôle et de maintenance du cytosquelette d'actine. mTOR1 inhibiteur de l'activation de l'autophagie peut-être facilement réprimée. En outre, lorsque mTOR1 est inhibé par une réduction du taux d'ATP, l'absence d'acides aminés ou la rapamycine (qui désactive la sensibilité de mTOR aux variations de nutriment), l'autophagie est activée (Wong *et al.*, 2015). Le lien entre le complexe mTOR2 et l'autophagie demeure peu connu. Lorsqu'il est stimulé, mTOR2 active l'expression de deux facteurs clés de l'autophagie : *light chain 3* (LC3B) et *BCL2/adenovirus E1B 19 kDa protein-interacting protein 3* (BNIP3) Le rôle le mieux caractérisé de mTOR2 est dans la régulation de la construction et le maintien du cytosquelette (Barth *et al.*, 2010).

L'activation indépendante de mTOR de l'autophagie est de plus en plus étudiée (Azad *et al.*, 2009). Par exemple, les molécules capables d'inactiver l'expression et l'activation des récepteurs de l'inositol et de l'inositol 1,4,5 triphosphate (IP3) activent l'autophagie indépendamment de mTOR (Fracchiolla *et al.*, 2018). L'autophagie est également activée directement par un mauvais repliement des protéines dans le réticulum endoplasmique via l'induction de l'expression du complexe ATG5-ATG12-ATG7 (Tasdemir *et al.*, 2007). Finalement, les EROs sont capables d'activer ATG4 et LC3B et ces effets, en situations normales, sont limités au niveau des mitochondries, principal organite à l'origine des ERO(Coto-Montes *et al.*, 2012).

L'autophagie débute par la formation du phagophore, une structure composée d'une double membrane formée par les facteurs Beclin-1 et les PI3K de classe III. Une fois mature, le phagophore génère l'autophagosome qui contient le cargo (un ensemble de structures qui seront libérées dans le lysosome) (Rubinsztein *et al.*, 2009). Toutes les étapes de l'autophagie et de liaison de l'autophagosome avec le lysosome sont réalisées par les *Autophagy-related genes* (ATGs), avec des fonctions conservées des levures aux mammifères (Y. Chen *et al.*, 2011). ATG7 est le lien entre la voie autophagique et la voie intrinsèque de l'apoptose. En situation d'hypoxie, ATG7 active ces deux voies de signalisation (B. Chen *et al.*, 2012a). ATG7 active également le facteur LC3B en LC3B-II en formant une liaison avec le phosphatidyléthanolamine, ATG12 et ATG5 sont responsables de la formation et de la maturation de l'autophagosome et le ATG16 est le responsable d'encrer le LC3B-II à

l'autophagosome (Coto-Montes *et al.*, 2012). Enfin, LC3B-II est actuellement la seule protéine qui a un rôle exclusivement dans l'autophagie. Quand l'autophagosome est mature, sa membrane externe fusionne avec le lysosome tandis que sa membrane interne et les composants du cargo, sont catabolisés par les cathepsines, enzymes lysosomales, en composants simples comme des acides gras, des acides aminés et des monosaccarides qui seront par la suite libérés dans le cytoplasme (Figure 6) (Pivtoraiko *et al.*, 2009).

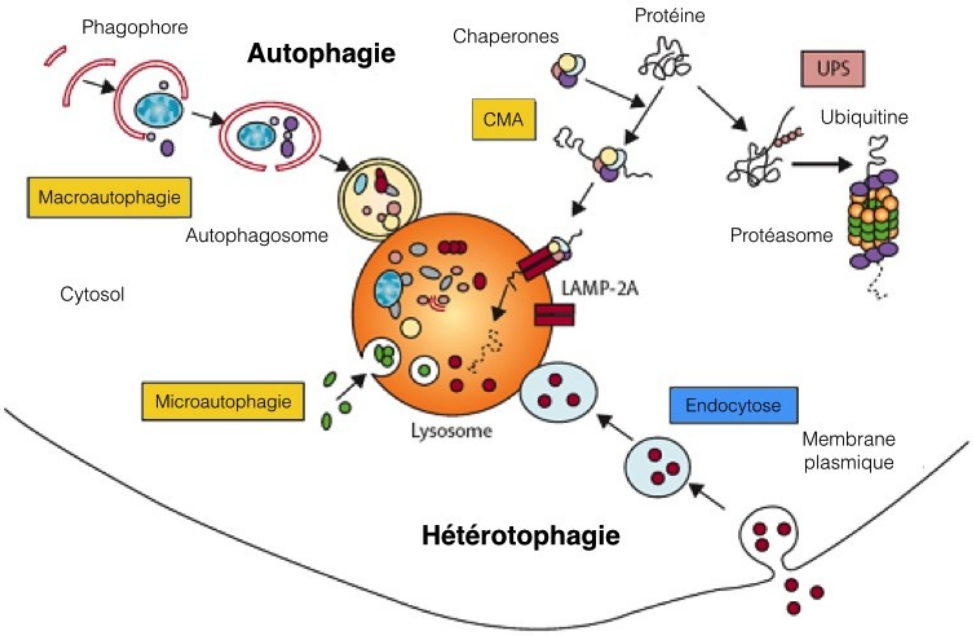

Figure 6: L'autophagie est une voie importante pour l'homéostasie trophoblastique. Les trois voies autophagiques : la macroautophagie, la microautophagie et l'autophagie médiée par les chaperonnes (CMA) sont actives dans les cellules trophoblastiques et adressent des composants endommagés aux lysosomes. Le système ubiquitine/protéasome (UPS, *ubiquitin-proteasome system*) est aussi un système de dégradation protéique actif et important à la survie cellulaire. L'hétérophagie est une voie de dégradation intracellulaire de matériaux originaires de la région externe de la cellule qui amène le *cargo* par pinocytose et endocytose. LAMP2A (*Lysosome associated membrane protein 2A*) Tirée de (Pivtoraiko *et al.*, 2009).

1.2.1. Autophagie et apoptose

De faibles taux d'acides aminés ou d'ATP ne sont pas uniquement des activateurs de l'autophagie ; ils activent également des mécanismes pro-inflammatoires et apoptotiques. Si les dommages cellulaires ne sont pas majeurs, l'autophagie permet le recyclage des macromolécules dans le but de restaurer l'homéostasie et d'inhiber l'apoptose (Cha *et al.*, 2014, He *et al.*, 2013). Dans ce contexte, l'autophagie joue un rôle de protection et de prévention de la

mort cellulaire. L'autophagie peut, d'autre part, dans des situations très rares chez les mammifères, promouvoir l'apoptose via une dégradation excessive et dérégulée des composants cellulaires par exemple en situation d'excès de stress oxydatif (Kroemer *et al.*, 2008). Dans ce cas l'activation des caspases associées à l'apoptose entraine le clivage de diverses protéines pro-autophagiques. Les fragments protéiques résultants de ces clivages acquièrent une nouvelle fonction pro-apoptotique, ce qui accélère (ou amplifie) l'activation de l'apoptose (Hung *et al.*, 2010, Y. Wang *et al.*, 2016)

La régulation mutuelle de l'autophagie et de l'apoptose est principalement contrôlée par la liaison de Beclin-1 avec Bcl-2. Lorsque Bcl-2 est lié à Beclin1 l'autophagie est inhibée. Dès que les taux d'ATP sont réduits, Bcl-2 et Beclin-1 se séparent et Beclin-1 est phosphorylé par le *Death associated protein kinase* (DAPK) pour initier la formation de l'autophagosome (Bildirici *et al.*, 2012, Debnath *et al.*, 2005, Kim *et al.*, 2007). L'autophagie augmente le seuil de stress requis pour l'induction de la mort cellulaire par plusieurs mécanismes. Ceci comprend l'élimination sélective des mitochondries endommagées, inducteurs potentiels de l'apoptose. L'autophagie peut, également, conduire à l'élimination sélective des transducteurs de signal pro-apoptotiques. Le dialogue entre l'autophagie et l'apoptose, tel qu'elle se produit dans une cellule ou entre des cellules distinctes, a un rôle crucial dans les processus physiopathologiques, y compris dans la grossesse normale et pathologique (Bildirici *et al.*, 2012, B. Chen *et al.*, 2012a, Y. Chen *et al.*, 2011, Klionsky *et al.*, 2016, Y. Wang *et al.*, 2016).

1.2.2. Autophagie et le trophoblaste villeux

Les effets protecteurs de l'autophagie tout au long de la grossesse ont été étudiés plus intensément au cours des dix dernières années. Tuuli et al. ont montré que l'autophagie et l'apoptose ont des actions simultanées dans les primocultures de vCTBs exposées à des conditions hypoxiques (Tuuli *et al.*, 2011). Des études de l'équipe du Dr Nelson ont décrit de façon approfondie les effets de l'hypoxie sur l'activation de l'autophagie à la fois chez les vCTBs (B. Chen *et al.*, 2012a) et le STB (Y. Wang *et al.*, 2016). Ils ont montré que la punicalagine (polyphénol du jus de grenade) aurait un rôle de protection dans la grossesse en agissant comme inhibiteur de l'apoptose et activateur de l'autophagie. Cependant, les mécanismes sous-jacents qui contrôlent la modulation différentielle de l'apoptose et de l'autophagie n'ont pas encore été étudiés.

En ce qui concerne les différentes étapes de la grossesse, il est évident que l'autophagie joue un rôle pivot en tant que voie protectrice. Pendant l'embryogenèse, l'activation

d'ATG7 et de LC3B permet la survie du blastocyste dans un modèle expérimental de souris présentant un retard d'implantation (J. E. Lee *et al.*, 2011). De même, pendant la différentiation de l'ovocyte en embryon, les protéines et les miARN maternels sont rapidement dégradés et remplacés par des protéines et des miARN zygotiques. La dégradation des protéines et des miARNs maternels par l'autophagie est réduite dans les souris homozygotes ATG5-knockout, entraînant des conditions sous-optimales pour le développement préimplantatoire (Tsukamoto *et al.*, 2008). Donc, l'autophagie peut induire le succès de l'embryogenèse même dans des contextes peu favorables.

L'environnement du evCTBs invasif au cours du premier trimestre est caractérisé par une condition hypoxique et pro-inflammatoire, ce qui induit l'activation de l'autophagie pour maintenir l'intégrité morphologique et fonctionnelle de la cellule. Les données allant dans le sens de l'activation de l'autophagie dans les trophoblastes invasifs sont basées sur des études réalisées avec des lignées cellulaires, qui sont de bons modèles, mais présentent des différences importantes d'expression génique par rapport aux cellules primaires (Novakovic *et al.* 2011). L'autophagie induite par l'hypoxie a été étudiée dans la lignée cellulaire HTR-8/SVneo dérivé de evCTBs (Choi *et al.* 2012). Dans ces cellules, l'inhibition de l'expression du facteur de transcription HIF-1α inhibe l'activation de l'autophagie et réduit le taux d'invasion. En outre, l'inhibition de l'autophagique au premier trimestre est corrélée à l'induction de l'endogline soluble (sENG), présente dans le sérum maternel des patients prééclamptiques (Nakashima *et al.* 2013). Donc, l'autophagie est nécessaire pour que l'invasion placentaire soit optimale et l'inhibition de l'autophagie augmente encore plus les effets délétères causés par des troubles de la grossesse (Nakashima *et al.* 2017).

L'autophagie est active dans le processus de syncytialisation des vCTBs en STB. Des vacuoles autophagiques ont été identifiées dans le trophoblaste villeux, et principalement dans le STB isolé de placentas de grossesses normales. Les placentas à terme provenant de patients prééclamptiques présentent une augmentation des vacuoles autophagiques, par rapport aux placentas de grossesses normotensives. Cette augmentation serait attribuée à un mécanisme de protection pour compenser des conditions sous-optimales dans le placenta (Nakashima *et al*. 2017; Oh *et al.* 2008). De même, les placentas provenant de patientes présentant une restriction de croissance intra-utérine présentent une augmentation de l'activation de l'autophagie par rapport aux placentas de donneurs sains (Curtis *et al*. 2013). Ces études suggèrent que le taux d'autophagique est considérablement plus bas dans les placentas de grossesses normales par rapport aux placentas de grossesses pathologiques, tels que la prééclampsie, la restriction de croissance intra-utérine et le diabète gestationnel

(Avagliano *et al.*, 2015).

1.3. Prééclampsie

La prééclampsie est la principale cause de mortalité fœtale et maternelle pendant la grossesse, avec une incidence mondiale de 6-8 %. Elle touche 18 % des femmes enceintes dans les pays moins riches, et est la cause de 15 % des accouchements prématurés dans les pays développés (Thornton *et al.*, 2013). Cette maladie de la grossesse, qualifiée de syndrome, est généralement diagnostiquée après la 20^{e} semaine de grossesse. Lorsqu'elle survient après la 34^{e} semaine de la grossesse (dans 80 % des cas), elle est considérée comme tardive, tandis que lorsqu'elle apparaît avant la 34^{e} semaine, on parle de prééclampsie précoce (Roberts *et al.*, 2009, Turner, 2010). En 2014 la société des obstétriciens et gynécologues du Canada a publié le nouveau guide pour la classification de la prééclampsie et le traitement à privilégier (Magee *et al.*, 2014). Dans le diagnostic de la prééclampsie, les mesures suivantes devraient être prises en considération soit une hypertension après la 20^{e} semaine de grossesse (tension artérielle systolique ≥ 140 mmHg et/ou pression artérielle diastolique ≥ 90) avec au moins un des effets suivants: une nouvelle protéinurie et/ou des altérations du système nerveux central (maux de tête, troubles visuels, convulsions), cardiorespiratoire (douleur thoracique, hypoxie, hypertension), hématologique (faible taux plaquettaire), rénal (créatinine élevée, acide urique élevé), hépatiques (douleur dans le quadrant supérieur droit, albumine plasmatique basse), et/ou fœto-placentaire (fréquence cardiaque fœtale anormale, oligohydramnios). La prééclampsie peut conduire à des complications sévères du système nerveux central (par ex. : éclampsie, syndrome d'encéphalopathie postérieure, cécité corticale, accident vasculaire cérébral, accident ischémique transitoire, déficit neurologique ischémique), cardiorespiratoire (hypertension sévère non contrôlée sur 12 heures malgré l'utilisation de trois antihypertenseurs, saturation en oxygène < 90 %, œdème pulmonaire, soutien inotrope positif ou ischémie myocardique ou infarctus), hématologique (numération plaquettaire < 50 × 10^{9}/L), rénal (indication d'hémodialyse); hépatique (rapport international normalisé (RIN) > 2 en l'absence de coagulopathie intravasculaire disséminée), et/ou fœto-placentaire (décollement placentaire avec évidence de compromission maternelle ou fœtale, canal inverse venosus A vague, ou mortinaissance).

Plusieurs facteurs de risque sont associés dans l'apparition de cette maladie, tels que l'obésité, la gestation multiple, l'hypertension chronique, les techniques de procréation médicalement assistée et la nulliparité. Concernant les deux derniers facteurs cités, ils sont

associés avec l'hypothèse immunitaire du déclenchement de la prééclampsie. Des études démontrent que cette maladie a une plus forte incidence chez les premières grossesses et que ce taux diminue avec le nombre de grossesses (Y. Y. Chen *et al.*, 2009, Skjaerven *et al.*, 2002). Un des activateurs de cette augmentation de la réponse immunitaire serait le sperme paternel, qui activerait une réponse inflammatoire classique. Plusieurs études épidémiologiques évoquent le fait qu'une exposition antérieure à la grossesse au sperme paternel (c.-à-d. du même partenaire) réduit significativement le risque de prééclampsie (Einarsson *et al.*, 2003).

1.3.1. Physiopathogenèse de la prééclampsie

Bien que la prééclampsie soit le plus souvent diagnostiquée à un stade avancé de la grossesse, les évènements moléculaires responsables de sa survenue apparaitraient dès le début de la grossesse (Ji *et al.*, 2013, Red-Horse *et al.*, 2004). Il est de l'avis général que des altérations du placenta et, plus spécifiquement, des trophoblastes seraient la cause principale de la prééclampsie. L'argument clef de cette affirmation est que le seul traitement définitif de la prééclampsie est l'élimination complète du placenta. D'ailleurs, quelques études démontrent qu'après l'accouchement, si le placenta persiste partiellement ou en totalité, les symptômes peuvent persister (Coppage *et al.*, 2002, Matsuo *et al.*, 2007)(Matsuo et al. 2007; Matthys et al. 2004; Coppage and Polzin 2002). Une des altérations observées dans les placentas prééclamptiques est un défaut d'invasion du myomètre utérin et du remodelage des artères spiralées utérines par les evCTBs (Handschuh *et al.*, 2007, Red-Horse *et al.*, 2004, Roberts *et al.*, 2009). Ceci a pour conséquence que les evCTBs ne réussissent pas à atteindre les vaisseaux utérins ni à remplacer correctement les cellules endothéliales, ce qui entraine un phénomène d'H/R (Crosley *et al.*, 2013). Ces altérations causées par la prééclampsie affectent fortement l'homéostasie placentaire. Morphologiquement, les placentas prééclamptiques sont de taille réduite avec un grand nombre de cellules trophoblastiques non différenciées (Figure 7).

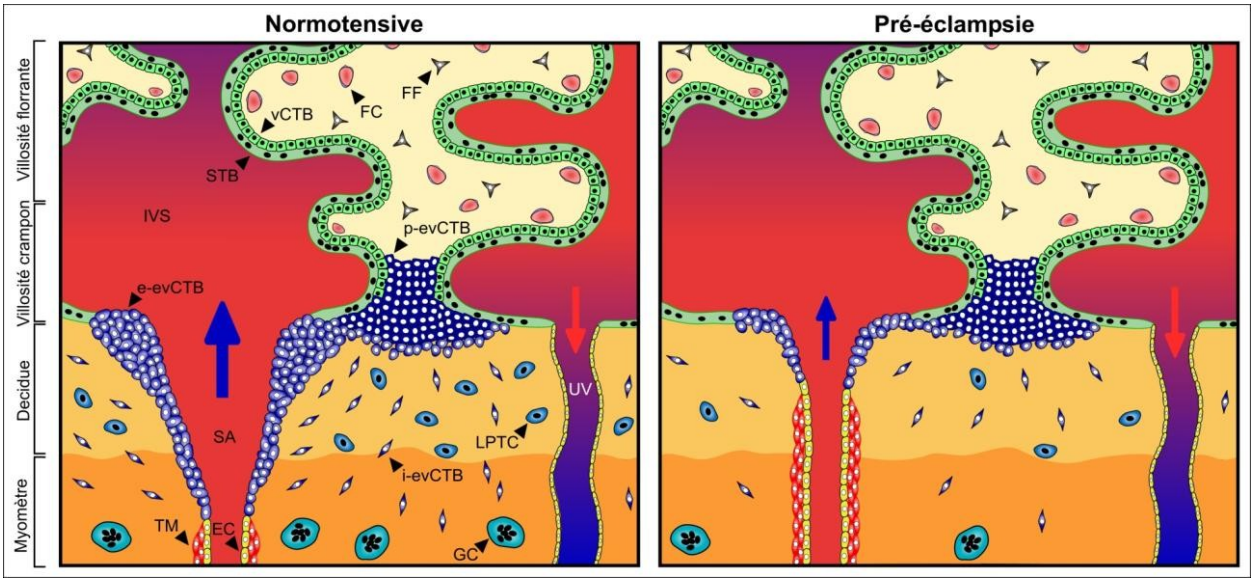

Figure 7: Comparaison de l'implantation placentaire dans une grossesse normale et prééclamptique. Modifié de (Lanoix D. *et al.*, 2012).

Durant la grossesse, le placenta produit plusieurs hormones et facteurs de croissances, qui sont sécrétés dans la circulation maternelle à des concentrations et sous différentes isoformes dans les cas de grossesse prééclamptique (Figure 8). Malgré le fait que de nombreux biomarqueurs ont été attribués aux placentas de grossesses prééclamptiques et malgré l'avancement des techniques de détection, il n'existe à l'heure actuelle aucun biomarqueur de la prééclampsie capable d'indiquer la présence de ce syndrome au début de la grossesse (c.-à-d. avant l'apparition des signes cliniques (Ji *et al.* 2013).

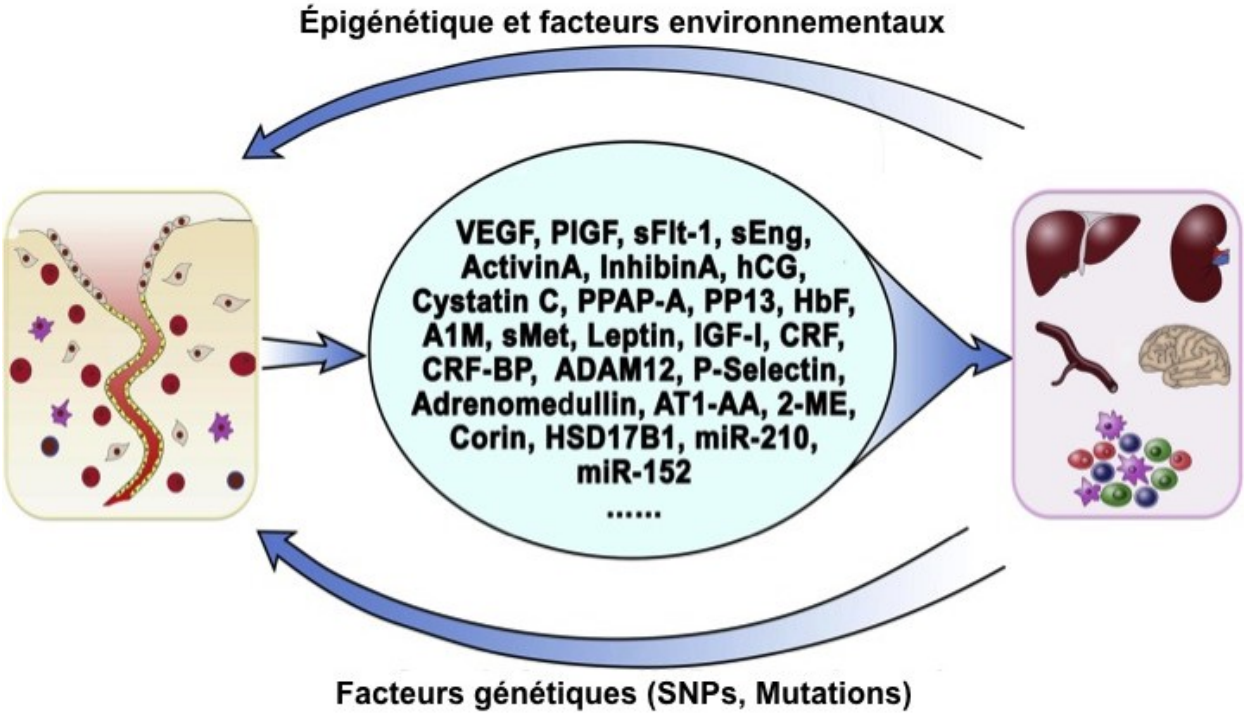

Figure 8: Les facteurs placentaires libérés par le placenta dans les grossesses prééclamptiques. Un mauvais développement et un défaut de l'apport sanguin placentaire génèrent la libération de plusieurs facteurs dans la circulation maternelle. Les taux anormaux de ces facteurs dans la circulation maternelle affectent plusieurs organes, comme le foie, les reins, les vaisseaux et le cerveau. Ces facteurs causent aussi des inflammations placentaire et systémique. Les facteurs génétiques, épigénétiques et environnementaux peuvent influencer les placentas endommagés. Modifié de (Ji *et al*. 2013).

Roberts et Hubel (2009) ont classifié l'échec du remodelage des artères spiralées utérines comme la première phase de la prééclampsie. La deuxième (et dernière) phase est caractérisée par les signes cliniques de cette maladie et par une augmentation du stress oxydatif, de l'inflammation et une augmentation de facteurs placentaires, comme l'*activin-A, l'Inhibin-A,* la leptine, le facteur de croissance placentaire et les microparticules trophoblastiques dans la circulation maternelle (Roberts *et al.*, 1999, Roberts *et al.*, 2009). L'augmentation du stress oxydatif peut survenir par deux situations distinctes : soit la quantité de sang est constamment faible et l'apport en oxygène est insuffisant pour garantir le bon développement fœto-placentaire; soit l'approvisionnement sanguin est intermittent provoquant un apport en oxygène variable provoquant une H/R (Ji *et al.* 2013; Roberts *et al.* 2009). Les variations d'oxygène dans le placenta de grossesse prééclamptique altèrent l'homéostasie du trophoblaste villeux. En outre, l'apoptose mitochondriale est augmentée dans les trophoblastes villeux exposés à une hypoxie (< 1 % d'O_2); ainsi que le p53 et le ratio Bax/Bcl-2 (Huppertz *et al.*, 2006, Lanoix *et al.*, 2013). *In vitro,* l'induction de l'apoptose par l'H/R dans les trophoblastes

villeux est inhibée par une exposition à la mélatonine (1 mM) ou à du jus de grenade (1% v/v) (B. Chen *et al.*, 2012b, Lanoix *et al.*, 2013). De plus, le STB est moins susceptible à l'activation de l'apoptose face à l'H/R, en comparaison aux vCTBs. La plus grande susceptibilité des vCTBs a généré l'hypothèse qu'il existe une interrelation inverse entre l'apoptose et l'autophagie dans le STB et que cette dernière est fondamentale au maintien du STB et à l'interface materno-fœtale (Chen *et al.* 2010; Tuuli *et al.* 2011). Contrairement à l'apoptose, l'autophagie associée à la prééclampsie a été peu étudiée (Bildirici *et al.* 2012; Klionsky *et al.* 2016). En 2008, Oh *et al.* ont démontré que les placentas de patientes atteintes de prééclampsie sévère exprimaient significativement plus de LC3B-II comparés aux placentas de grossesse normale. *In vitro,* ils ont exposé les cellules JEG-3 (lignée de choriocarcinome placentaire humain, présentant un phénotype similaire aux evCTBs) à des conditions hypoxiques ou au TNF. En condition hypoxique (>1% O_2) l'expression de Beclin-1 est significativement diminuée tandis que LC3B-II est augmenté en comparaison aux cellules exposées au TNF-α ou sous 20% O_2 (contrôles) (Oh *et al.*, 2008). D'autre part, 24 heures d'hypoxie induisent l'activation de l'autophagie dans les vCTBs *in vitro* via l'inhibition de mTOR1. L'inhibition de ATG7 par la stratégie de siARN inhibe les voies apoptotiques et autophagiques dans le vCTB (B. Chen *et al.*, 2012a, Wullschleger *et al.*, 2006). La coexposition d'explants de trophoblaste villeux aux vitamines E et C et à l'hypoxie module aussi l'autophagie et l'apoptose. L'expression du LC3B-II est augmentée alors que le taux de Bcl-2 est diminué dans les explants exposés aux à l'hypoxie (Hung *et al.*, 2010). Ces données suggèrent que la mélatonine joue un rôle de protection dans le trophoblaste placentaire face à des conditions de grossesse sous-optimales, tel que l'H/R, notamment par la régulation de l'autophagie.

1.4. Mélatonine

Tout d'abord découverte dans la glande pinéale, la mélatonine produite par cette glande joue un rôle dans la régulation du cycle circadien et circannuel. Depuis, plusieurs travaux ont démontré la présence, la production et un rôle de la mélatonine dans différents tissus et cellules, tels que tractus gastro-intestinal, la peau, la rétine et le système périphérique reproductif comme l'ovaire, le liquide amniotique et le placenta (Lanoix *et al.*, 2008, A. Slominski *et al.*, 2008, R. M. Slominski *et al.*, 2012).

La mélatonine est synthétisée à partir de l'acide aminé essentiel L-tryptophane qui est d'abord transformé en sérotonine, puis convertie en N-acétyl-5-méthoxytryptamine (mélatonine) par l'activité séquentielle de deux enzymes spécifiques AANAT suivie de l'acétylsérotonine-O-méthytransférase (ASMT ou HIOMT; hydroxyindole-O-méthyltransférase), qui méthyle le produit de l'AANAT en mélatonine (Tan *et al.*, 2013, Wurtman *et al.*, 1968). En parallèle à des actions directes, la mélatonine joue un rôle important dans l'homéostasie cellulaire via l'activation de ces récepteurs, MT1 et MT2 (Tan *et al*. 2013).

Les récepteurs de la mélatonine MT1 et MT2 font partie de la famille des récepteurs à sept domaines transmembranaires (aussi appelés récepteurs couplés aux protéines G (RCPG)) (Dubocovich *et al.*, 2005, Dubocovich *et al.*, 2003). L'activation de MT1 et MT2 est impliquée dans la régulation de différentes voies de signalisation selon le tissu ou la cellule : AMPc/PKA, PLC, PLA2 et les canaux K^+ et Ca^{2+} (Tableau 2) (Dubocovich *et al.*, 2005, Dubocovich *et al.*, 2003). Leurs structures sont similaires avec une homologie de séquence en acides aminés de 60 % (Al-Ghoul *et al.*, 1998, Dubocovich *et al.*, 1998, Masana *et al.*, 2001).

La mélatonine est connue comme un neutralisateur de radicaux libres, un effet récepteurs-indépendants et -dépendants. Il y a plusieurs caractéristiques qui font de la mélatonine un protecteur de la viabilité et de l'homéostasie cellulaire extrêmement efficace. Premièrement, la mélatonine peut atteindre tous les compartiments subcellulaires ; elle est présente en grande concentration dans les mitochondries, le noyau et la membrane cytoplasmique, en fait la mitochondrie est un des sites de production de la mélatonine (Slominski *et al.* 2012). Cette indolamine est très hydrophile et lipophile, et est donc rapidement absorbée et traverse toutes les barrières morphologiques, comme le placenta et la barrière hématoencéphalique. Cette caractéristique est essentielle pour sa bonne distribution et son efficacité en tant qu'antioxydant (da Silva *et al.*, 2011, Galano *et al.*, 2013, Reiter *et al.*, 2013). En effet, Milczareck *et al.* (2010) ont comparé l'effet de la mélatonine et des vitamines C et E et observé la supériorité de la mélatonine comme molécule antioxydante due à son large spectre de neutralisation des EROs (Milczarek *et al.*, 2010). Deuxièmement, les métabolites de la mélatonine générés par la neutralisation des radicaux libres, comme le *N^1-acetyl-5-methoxykynuramine* (AMK), ont également des propriétés antioxydantes ; certains d'entre eux sont même plus actifs que la mélatonine (Figure 9). Enfin, la mélatonine exerce aussi des effets antioxydants via l'activation de ses récepteurs MT1 et MT2 qui induisent l'expression et l'activité des enzymes antioxydantes (Reiter *et al*. 2013).

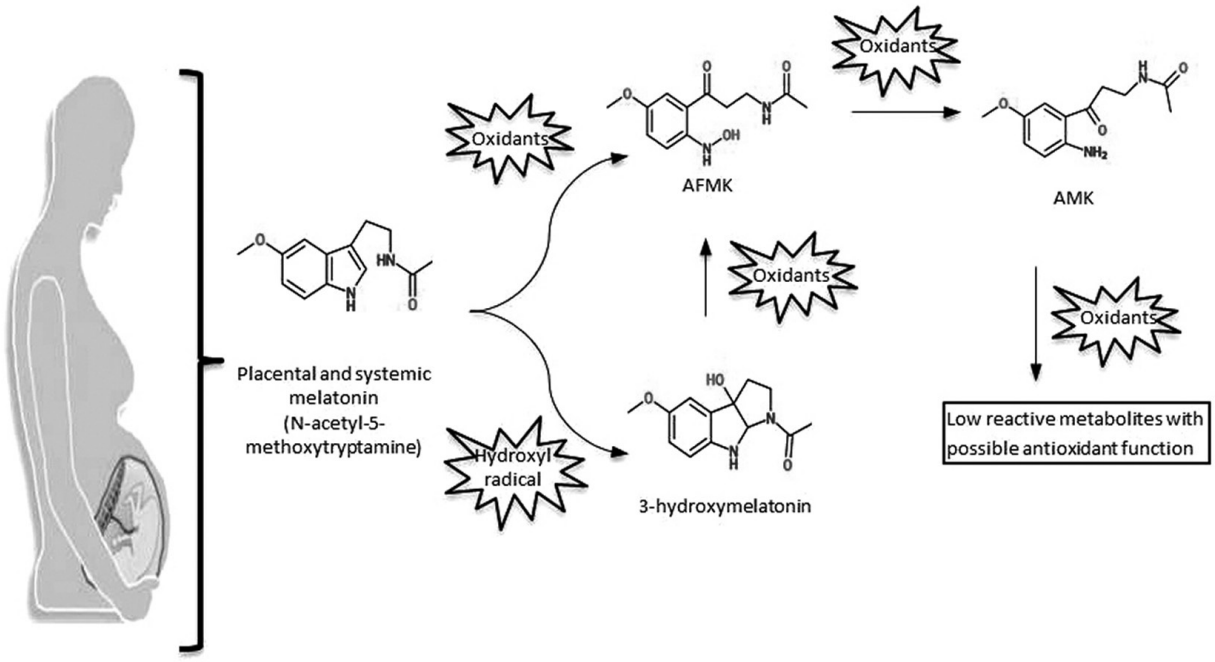

Figure 9: Cascade antioxydante de la mélatonine. L'activité antioxydante de la mélatonine est décrite dans plusieurs organes, parmi eux le placenta. Les métabolites de la mélatonine ont des actions antioxydants (capteurs de radicaux libres). AFMK: N2-acétyl N2-formyl-5-méthoxykynuramine, AMK: N1-acétyl-5-méthoxykynuramine (Voir Annexe 3 (Sagrillo-Fagundes *et al.*, 2014)).

La mélatonine a également des propriétés anti-inflammatoires. Entre autres, elle module et diminue l'expression et l'activité de la forme inductible de l'oxyde nitrique synthase (iNOS) et de la cyclooxygénase (COX-2) (Carrillo-Vico *et al.*, 2005a, Carrillo-Vico *et al.*, 2005b, Carrillo-Vico *et al.*, 2005c). Elle inhibe également l'activation du *nuclear factor κB* (NFκB), un facteur clé dans les maladies inflammatoires. D'autre part, l'augmentation des taux de NFkB et du TNF réduisent la transcription de l'arylalkylamine N-acetyltransférase (AANAT), enzyme limitant dans la synthèse de la mélatonine (Markus *et al.*, 2017, Muxel *et al.*, 2016).

Tableau 2: Les voies de signalisation modulées par la mélatonine et ses récepteurs

Voie de signalisation	Récepteurs impliqués	Effet de la mélatonine	Effets	Références
AMPc/PKA	MT1	Inhibition	Inhibition de la sécrétion d'insuline	(Fustin *et al*. 2009)
PLC	MT1 et MT2	Activation	Induit la différentiation cellulaire	(Fukunaga *et al*. 2002)
ERK1/2	MT1 et MT2	Inhibition	Réduction de la croissance tumorale	(Blask *et al*.2002)
PLA2	MT1 et MT2	Activation	Contrôle inflammatoire intracellulaire dans les leucocytes	(Radogna *et al.* 2009)
Canaux K^+	MT1	Activation	Contractilité des vaisseaux sanguins	(Nelson *et al.* 2001)

AMPc/PKA : Cyclic adenosine monophosphate- dependent protein kinase ; PLC : Phospholipase C ; ERK 1/2 : extracellular signal-regulated kinase 1/2 ; PLA2 : Phospholipase A2.

Le MT3, que l'on croyait être un troisième récepteur de la mélatonine, est en fait l'enzyme quinone réductase 2 (NQO2) possédant un site de liaison pour la mélatonine. Le transcrit de NQO2 n'est pas exprimé dans le placenta humain (Nosjean *et al.*, 2000). La mélatonine se lierait aussi aux récepteurs nucléaires du groupe ROR/RZR (*retinoid orphan receptor et retinoid Z receptor*). Malgré la présence des récepteurs ROR/RZR dans les cellules placentaires, récemment il a été prouvé que la mélatonine ne se lie pas à ces récepteurs (Slominski, *et al* 2014).

1.4.1. Rôle dans la survie cellulaire

Plusieurs études démontrent l'implication de la mélatonine dans la régulation de la survie cellulaire. Elle régule la voie apoptotique via ses récepteurs et directement via ses propriétés antioxydantes. L'action via les récepteurs MT1 et MT2 entraîne une augmentation de l'expression et de l'activité des enzymes antioxydantes, une inhibition de la phosphorylation de la MAPK p38 et de c-Jun N-terminal (JNK), ce qui conduit à l'inhibition de la protéine pro-apoptotique p53, facteur clé de l'activation de l'apoptose par la voie mitochondriale (Carrillo-Vico *et al.*, 2005c, Coto-Montes *et al.*, 2012, da Silva *et al.*, 2011). La liaison entre la mélatonine

et ses récepteurs stimule aussi la voie PI3K/AKT, ce qui empêche la translocation de la protéine BAD du cytoplasme vers l'intérieur de la mitochondrie et l'apoptose, et donc maintien la survie cellulaire (Choi *et al.*, 2008). Spécifiquement dans la mitochondrie, la mélatonine augmente l'activité des complexes de la chaîne de transport d'électrons (ETC) avec comme conséquence une augmentation d'ATP. Cette indolamine réduit aussi la lipopéroxydation de la membrane interne et la libération d'espèces réactives de l'ETC et conséquemment empêche la perméabilisation de la membrane mitochondriale (Coto-Montes *et al.*, 2012)(Cardinali et al. 2013).

La mélatonine a des effets distincts dans les cellules normales et les cellules tumorales (pour revue voir (Reiter *et al.* 2017)). Notre groupe a démontré des effets différents de la mélatonine dans les cellules trophoblastiques primaires versus les cellules issues d'un choriocarcinome placentaire, les cellules BeWo (Lanoix *et al.*, 2012b). Cette étude a démontré que la mélatonine induit la survie cellulaire des trophoblastes primaires, mais induit la mort des cellules BeWo, via des effets contraires sur l'apoptose mitochondriale. Plusieurs études ont montré des actions antitumorales de la mélatonine seule ou en complément avec d'autres thérapies (Lopes *et al.*, 2016, Maschio-Signorini *et al.*, 2016). Entre autres, des études démontrent que la mélatonine peut moduler la survie cellulaire en contrôlant l'autophagie des cellules normales et tumorales (Tableau 3) (Lanoix *et al.*, 2012b, Lopes *et al.*, 2016, Maschio-Signorini *et al.*, 2016).

Tableau 3: Ensemble des effets de la melatonin sur les voies qui modulent l'autophagie.

Modèle expérimental	Cible	Effet sur l'autophagie	Méchanisme d'action	Effet sur les cellules
Glioblastome malin (Zhang *et al.* 2015)	Cellules de glioblastomes malin	↑	Inhibition de la voie Akt	↑ mort cellulaire
Neurotoxicité induite par l'arsenite (Teng *et al.* 2015)	Primoculture des neurones corticaux de rat	↓	–	↓ mort cellulaire
Neurotoxicité induite par le cadmium (Li *et al*. 2016)	Cellules de neuroblastome de souris	↑	↑ expression protéique du lysosome	↓ mort cellulaire
Neurotoxicité induite par l'acide kaïnique (Chang *et al.* 2012)	Tissus hippocampiques de souris	↓	–	↓ mort cellulaire
Neurotoxicité induite par la méthamphé-tamine (Kongsuphol *et al*. 2009)	Cellules SK-N-SH	↓	↓ mTOR et ↓de l'activité de 4EBP1	↓ mort cellulaire
Neurotoxicité induite par la rotenone (Zhou *et al.* 2012)	Cellules Hela	↓	↓ Bax	↓ mort cellulaire
Neurotoxicité médiée par le prion (Jeong *et al.* 2015)	Lignée de neuroblastome humain (SH-SY5Y)	↑	↑ α7nAchR	↓ mort cellulaire
Lésion cérébrale traumatique (Lin *et al.* 2016)	Cortex des rats	↑	↓ mTOR	↓ mort cellulaire
H/R néonatal (Hu *et al.* 2017)	Hippocampe, cortex cérébral et cellules PC12	↓	–	↓ mort cellulaire
Vieillissement (Nopparat *et al.* 2017)	Cellules SH- SY5Y	↑	↑ SIRT1 ↓ NFκB	↓ mort cellulaire
Conservation de greffe de foie gras (Zaouali *et al.* 2013)	Foies stéatosiques	↑	↑ AMPK	↓ mort cellulaire
Cancer du foie (Ordonez *et al.* 2015)	Cellules HepG2	↑	Phosphorylation de JNK	↑ mort cellulaire
Carcinome hépatocellulaire (Liu *et al.* 2012)	Lignée d'hépatome de souris H22	↑	↓ mTOR ↑ Akt	↓ mort cellulaire
Obesité (de Luxan-Delgado *et al.* 2014)	Foie de souris ob/ob	↓	↑ p62 ↓ PPAR-γ	Adiposité réduite
Fibrose hépatique (San-Miguel *et al.* 2015)	Foie de souris	↓	↓ mTOR	Protection contre la fibrose
H/R du foie (Kang *et al.* 2014)	Foie des souris C57BL/6	↓	↑ mTOR ↑ 4EBP1	↓ mort cellulaire
Hépatotoxicité induite par le cadmium (Pi *et al.* 2015)	Foie des souris C57BL/6	↓	↑ Sirt1, ↑ Sirt3 ↑activite de SOD SOD2	↓ mort cellulaire

Akt: Protein kinase B (PKB); mTOR (mammalian target of rapamycin); 4EBP1 (Eukaryotic translation initiation factor 4E-binding protein 1); Bax (Bcl-2-associated X protein); α7nAchR (alpha-7 nicotinic receptor); SIRT1 (Sirtuin 1); NFκB (Nuclear factor-κB); AMPK (5' adenosine monophosphate-activated protein kinase); JNK (c-Jun N-terminal kinase); PPAR-γ (Peroxisome proliferator-activated receptor gamma); SOD2 (Superoxide dismutase 2).

Il a été démontré que les actions antioxydantes de la mélatonine renforcent les effets protecteurs de l'autophagie, afin de rétablir l'homéostasie face à des conditions pathologiques. Les récepteurs MT1 et MT2, d'un autre côté, seraient capables de moduler les facteurs

d'autophagie en amont pour optimiser ses actions (Figure 10) (Roohbakhsh *et al.* 2018; Ordonez *et al.* 2015; de Luxan-Delgado *et al.* 2016; Asghari *et al.* 2018)

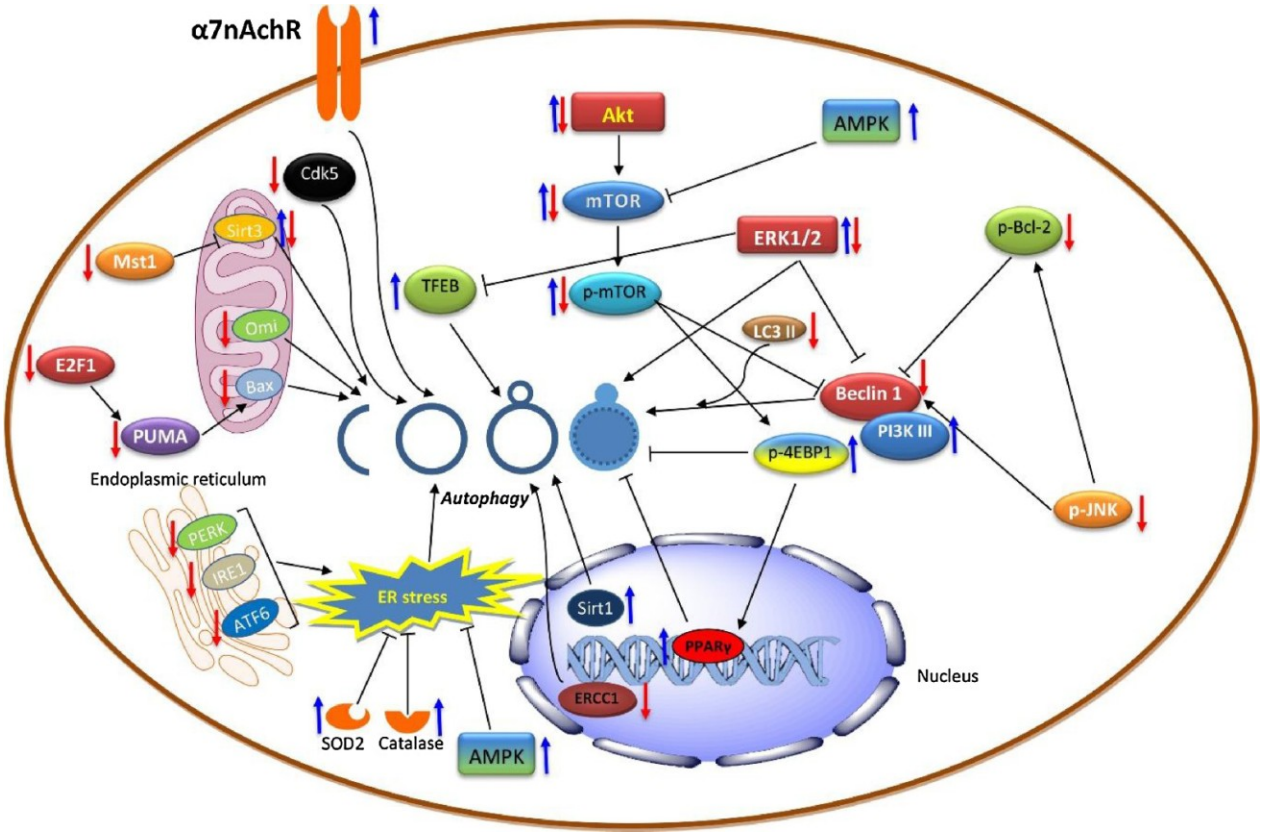

Figure 10: Mélatonine et autophagie. La mélatonine via différentes cibles supprime ou active l'autophagie en fonction du modèle expérimental, du type de cellule et de nombreux autres paramètres. Dans nombreuses études, la mélatonine induit des effets protecteurs et bénéfiques soit par suppression, soit par activation de l'autophagie. A7nAchR : α7 nicotinic acetylcholine receptor; Cdk5 : Cell division protein kinase 5; Sirt3 : sirtuine 3; Mst1 : macrophage stimulating 1; Omi/HTRA2 (HtrA serine peptidase 2); Bax : bcl-2-like protein 4; PUMA : p53-upregulated modulator of apoptosis; PERK : protein kinase R (PKR)-like endoplasmic reticulum kinase; IRE1 : inositol-requiring enzyme 1; ATF6 : Activating transcription factor 6; TFEB : Transcription factor EB; AMPK : 5' AMP-activated protein kinase; mTOR : mammalian target of rapamycin; ERK1/2 : extracellular signal–regulated kinases; Bcl-2 : B-cell lymphoma 2; JNK : c-Jun N-terminal kinase; PI3K : Phosphatidylinositol-4,5-bisphosphate 3-kinase; 4EBP1 : Eukaryotic translation initiation factor 4E-binding protein 1; PPAR-γ : Peroxisome proliferator-activated receptor gamma; ERCC1 : excision repair cross-complementation group 1; Sirt1 : Sirtuin 1; ↑, active; ↓, inhibe. Tirée de (Roohbakhsh *et al.*, 2018).

1.4.2. Mélatonine et grossesse normale

Le placenta humain produit la mélatonine et exprime ses récepteurs au cours de toute la grossesse (Lanoix *et al.*, 2008, Soliman *et al.*, 2015). En 2005, Iwasaki *et al.* ont démontré

l'expression de l'ARNm des enzymes de synthèse de la mélatonine (AANAT et ASMT) dans le tissu placentaire issu du premier trimestre de la grossesse (Iwasaki *et al.*, 2005). La confirmation de la présence de ces enzymes, des récepteurs MT1 et MT2 et de la mélatonine dans le placenta tout au long de la grossesse a été démontrée par notre laboratoire (Soliman *et al.* 2015; Lanoix *et al.* 2008). Les taux plasmatiques maternels de mélatonine augmentent progressivement au cours de la grossesse avec un pic maximal au moment de l'accouchement. Les femmes enceintes voient donc leurs concentrations de mélatonine plasmatiques (diurnes et nocturnes) augmenter par rapport aux femmes non enceintes (Y. Nakamura *et al.*, 2001, Tamura *et al.*, 2008). Ces données, et la diminution des taux de mélatonine après la délivrance du placenta (Figure 11), suggèrent que la mélatonine placentaire serait la source de l'augmentation de mélatonine plasmatique chez les femmes enceintes. De plus, certains auteurs suggèrent que l'augmentation de mélatonine à terme est impliquée dans le déclenchement de l'accouchement. La mélatonine et l'ocytocine stimulent respectivement le récepteur MT2 (MT2R) et le récepteur d'ocytocine (OTR) ce qui entraîne une augmentation de la disponibilité de l'actine pour la liaison à la myosine et l'induction de la contractilité. Ces deux hormones activent la voie PLC/PKC dans les cellules du myomètre (Sharkey *et al.* 2009; Sharkey *et al.* 2010). L'importance de cette interaction a été mise en évidence par des études démontrant que la contractilité des cellules myométriales *in vitro* est activée lorsqu'elles sont cotraitées avec l'ocytocine et la mélatonine, ce phénomène n'est pas observé si les cellules sont traitées seulement avec l'ocytocine (Sharkey, Cable, and Olcese 2010; Sharkey et al. 2009; Reiter, Tan, et al. 2013). Ces études pourraient expliquer que les accouchements sont plus nombreux pendant la période nocturne, aussi bien pour les accouchements à terme que pour les prématurés (Reiter *et al.* 2013; Vatish *et al.* 2010).

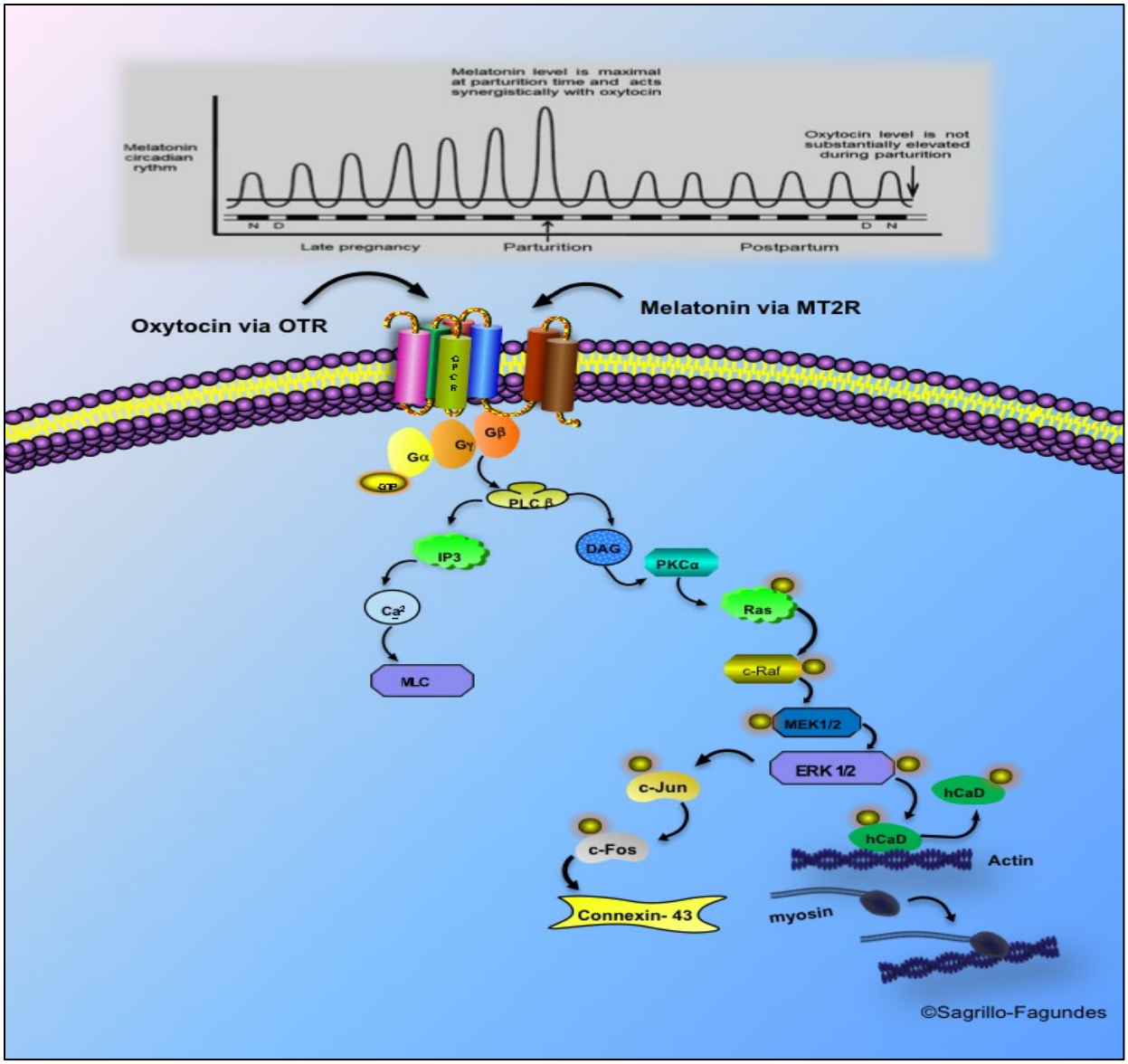

Figure 11: Modèle d'action synergique de la mélatonine et de l'ocytocine sur la contractilité myométriale au moment de l'accouchement. Le rythme circadien de la mélatonine et le taux d'ocytocine sont décrits au cours du dernier trimestre de la grossesse, au moment de l'accouchement et après la naissance. La mélatonine et l'ocytocine stimulent respectivement le récepteur MT2 (MT2R) et récepteur d'ocytocine (OTR) pour activer de manière synergique PLCβ, qui hydrolyse PIP2 en DAG et IP3. DAG active PKCα, activant la voie de signalisation Ras/Raf/MAPK et conduisant à la phosphorylation de hCaD. Ce augmente la disponibilité de l'actine pour la liaison à la myosine et l'induction de la contractilité. De plus, PLCβ stimule à la fois c-Jun et c-Fos, résultant en une communication jonctionnelle améliorée par l'activation de la connexine 43. Enfin, IP3 stimule la libération de calcium qui active la MLC et améliore la contractilité myométriale. PLC : phospholipase, PIP2 : phosphatidylinositol 4,5-bisphosphate, DAG : diacylglycérol, IP3 : inositol 1, 4, 5-triphosphate, PKC : protéine kinases de type C, MAPK : mitogenactivated protein kinase, hCaD : caldesmon, MLC : chaîne légère de myosine (voir Annexe 3 (Sagrillo-Fagundes *et al.* 2014)).

1.4.3. Mélatonine et grossesses pathologiques

Il existe plusieurs études suggérant que la mélatonine joue un rôle dans la physiopathologie ou l'étiologie de la prééclampsie. Cette maladie de la grossesse est l'une des principales causes de morbi-mortalité maternelle et fœtale et d'accouchements prématurés (décrite en détail à la section 1.4.) (Cartwright *et al.* 2010;Ji *et al.* 2013; Lanoix *et al*. 2012; Reiter *et al*. 2013). Une des hypothèses pour expliquer les signes cliniques de cette maladie est une mauvaise implantation placentaire causant une H/R et une augmentation du stress oxydatif. Comme décrit précédemment, la mélatonine est un puissant antioxydant avec des effets plus puissants que les vitamines C et E sur plusieurs tissus dont le placenta (Milczarek *et al*. 2010; Reiter *et al.* 2013). Notre groupe a évalué l'effet d'une exposition à la mélatonine sur des primoculture de STB exposé à l'H/R. Les résultats démontrent un effet protecteur de la mélatonine. La mélatonine renverse les effets inhibiteurs de l'H/R sur l'expression et l'activité des enzymes antioxydantes et réduit l'activation des facteurs de transcription redox-sensitifs (NFκB et HIF-1) induit par l'H/R (Lanoix *et al.* 2013) Ces facteurs de transcription sont impliqués dans l'activation de l'inflammation dans le cas de grossesse prééclamptique, mais l'effet de la mélatonine au niveau inflammatoire n'a jamais été étudié dans le trophoblaste. La mélatonine renverse aussi l'effet stimulateur de l'H/R sur l'expression des facteurs responsables de l'activation de l'apoptose intrinsèque, tels que PARP, ROCK-1 et les caspases 9 et 3. Enfin, la mélatonine rétablie le ratio Bax/BcL-2 en renversant les effets de l'H/R sur l'expression de Bcl-2 dans les primocultures de vCTBs (Lanoix *et al*. 2013). Notre équipe a également démontré que la prééclampsie est associée avec une diminution de l'activité et de l'expression de AANAT, de la concentration de la mélatonine et de l'expression des récepteurs MT1 et MT2 dans le tissu placentaire (Lanoix *et al.*, 2012a). Yamamoto *et al.* (2013) ont montré que la prééclampsie augmente l'expression des récepteurs MT1 et réduit l'expression du tryptophane hydrolase (Tph) dans le placenta, la première enzyme dans la voie de synthèse de la mélatonine à partir du L-tryptophane, par contre les niveaux de mélatonine placentaire n'ont été pas mesurés (Yamamoto *et al.*, 2013).

La restriction de croissance intra-utérine et la prééclampsie sont intimement liées : les deux sont associées avec une placentation et une invasion utérine altérée au cours des premières semaines de la grossesse. Cette pathologie est la deuxième cause de morbimortalité périnatale (Lausman *et al.*, 2013, Lausman *et al.*, 2012). Comme le démontre élégamment Nakamura et al., comme la prééclampsie, la restriction de croissance intra-utérine est associée à une diminution des taux de mélatonine sanguin maternelle (Y. Nakamura *et al.*, 2001). Cette

maladie de la grossesse est également associée à une réduction des taux urinaires de 6-sulfatoxymélatonine (métabolite de la mélatonine) chez les nourrissons (Tableau 4) (Kennaway *et al.*, 2001).

Tableau 4 : Le rôle de la mélatonine sur les grossesses normales et sur les grossesses pathologiques

État de la grossesse	Taux maternel de mélatonine	Taux placentaire de mélatonine	Expression des récepteurs MT1/MT2 dans le placenta	Effet de la mélatonine exogène
Grossesse sans maladie	↑ des valeurs au cours de la grossesse (sérum) : maximum à l'accouchement (1)	↑expression de l'enzyme de synthese AANAT au 3^{e} trimestre (2)	MT1 :↑ au 1er trimestre MT2 : stable pendant la grossesse (2)	↑ sérum maternel et dans les vaisseaux fœtales (3 & 5)
Prééclampsie	↓par rapport les grossesses sans maladie (1)	↓ par rapport les grossesses sans maladie (4)	MT1 : ↓ MT2 : ↓ (4)	↓ taux de prématurité par rapport les grossesses sans maladie (5)
Restriction de croissance intra-utérine	Taux comparable aux grossesses sans maladie (1)	Pas de données	Pas de données	Essai clinique en cours (6)
Diabètes mellitus gestationnel	↓ par rapport les grossessez sans maladie (salive) (7)	Pas de données	↑ Polymorphisme génétique du MT2 (8)	Pas de données

(1) : (Nakamura *et al*. 2001); (2) : (Soliman *et al.* 2015); (3) : (Okatani *et al.*, 1998); (4) : (Lanoix *et al*. 2012); (5) : (Hobson *et al.*, 2018); (6) : (Miller *et al.*, 2014); (7) : (Shimada *et al.*, 2016); (8) : (C. Li *et al.*, 2018)

La diminution de production de mélatonine observée dans les grossesses compliquées par une H/R placentaire (e.g. prééclampsie, restriction de croissance intra-utérine) pourrait altérer le développement du fœtus. En outre, la restriction de croissance intra-utérine affecte le développement de plusieurs organes et systèmes et est associée à des altérations du neurodéveloppement et autres complications à long terme (Karowicz-Bilinska *et al.*, 2007). Il a été démontré pendant la période embryonnaire que la mélatonine protège le cerveau des rats contre le stress oxydatif, la lipopéroxydation et les dommages à l'ADN causés par une ischémie/réperfusion (Wakatsuki *et al.*, 2000). D'autre part, la pinéalectomie chez des rattes gestantes, qui entraîne une suppression de la sécrétion de mélatonine pinéale et du cycle circadien, est associée à des altérations comportementales chez les nouveau-nés, qui se rétablissent avec un traitement à la mélatonine après la naissance (Sagrillo-Fagundes *et al.*, 2016).

Des traitements chroniques ou aigus avec des doses élevées de mélatonine n'ont démontré aucune toxicité dans plusieurs modèles expérimentaux. En outre, aucune toxicité même à des doses massives (200 mg/kg/jour) de mélatonine n'a été observée dans plusieurs modèles expérimentaux de la grossesse (Arendt, 1997, Zawilska *et al.*, 2009). Par contre, une étude récente dans un modèle de restriction de la croissance intra-utérine chez la brebis a observé des effets indésirables de la mélatonine. Dans cette étude les brebis gestantes maintenues en haute altitude (3600 m au-dessus du niveau de la mer), soit en condition hypoxique, ont été traitées pendant les 50 derniers jours de la grossesse avec 10 mg/kg/jour de mélatonine. Dans ce modèle animal, le *statut* antioxydant maternel (250 %) et la durée de la gestation (7,5%) étaient augmentés dans les animaux du groupe mélatonine par rapport au groupe témoin et le traitement à la mélatonine a diminué la biométrie néonatale et le poids à la naissance (-25 %). Malheureusement, aucune étude plus approfondie n'a été menée pour étudier les mécanismes impliqués (Gonzalez-Candia *et al.*, 2016). Comme discuté par les auteurs, il s'agit d'un modèle unique, qui a révélé des résultats contradictoires par rapport à une pléthore d'études utilisant une variété de modèles animaux pour étudier le potentiel de la mélatonine approche préventive et thérapeutique dans les cas de restriction de la croissance intra-utérine (pour revue voir (Sagrillo-Fagundes *et al.* 2016)).

Des essais cliniques avec la mélatonine ont récemment été amorcés dans le traitement de la prééclampsie et de la restriction de la croissance intra-utérine (Hobson *et al.* 2013; Miller *et al.* 2014). Les résultats de l'essai clinique de phase I concernant la sécurité et l'efficacité de la mélatonine dans ces deux pathologies sont prometteurs (Hobson *et al.* 2018). Dans cette étude, 20 femmes enceintes diagnostiquées avec une prééclampsie précoce ont été traitées trois fois par jour avec 10 mg de mélatonine. Un groupe de 40 femmes enceintes diagnostiquées avec une prééclampsie sans traitement avec mélatonine a été adopté comme groupe témoin. Aucun effet néfaste de toxicité n'a été détecté ni pour la mère et ni pour le bébé dans le groupe traité avec la mélatonine. Tous les bébés du groupe mélatonine présentent une réduction du poids à la naissance, comparé à 77% des nouveau-nés pour le groupe témoin. Par contre, le traitement avec la mélatonine a réduit la nécessité de traitement antihypertenseur. Plus important encore, la mélatonine a réduit le taux d'accouchement prématuré, prolongeant la grossesse de six jours. Une augmentation suffisante pour réduire la morbidité et mortalité périnatale (Hobson *et al.* 2018). Les effets bénéfiques potentiels de la mélatonine exogène dans le traitement de grossesse pathologique sont présentés à la Figure 12.

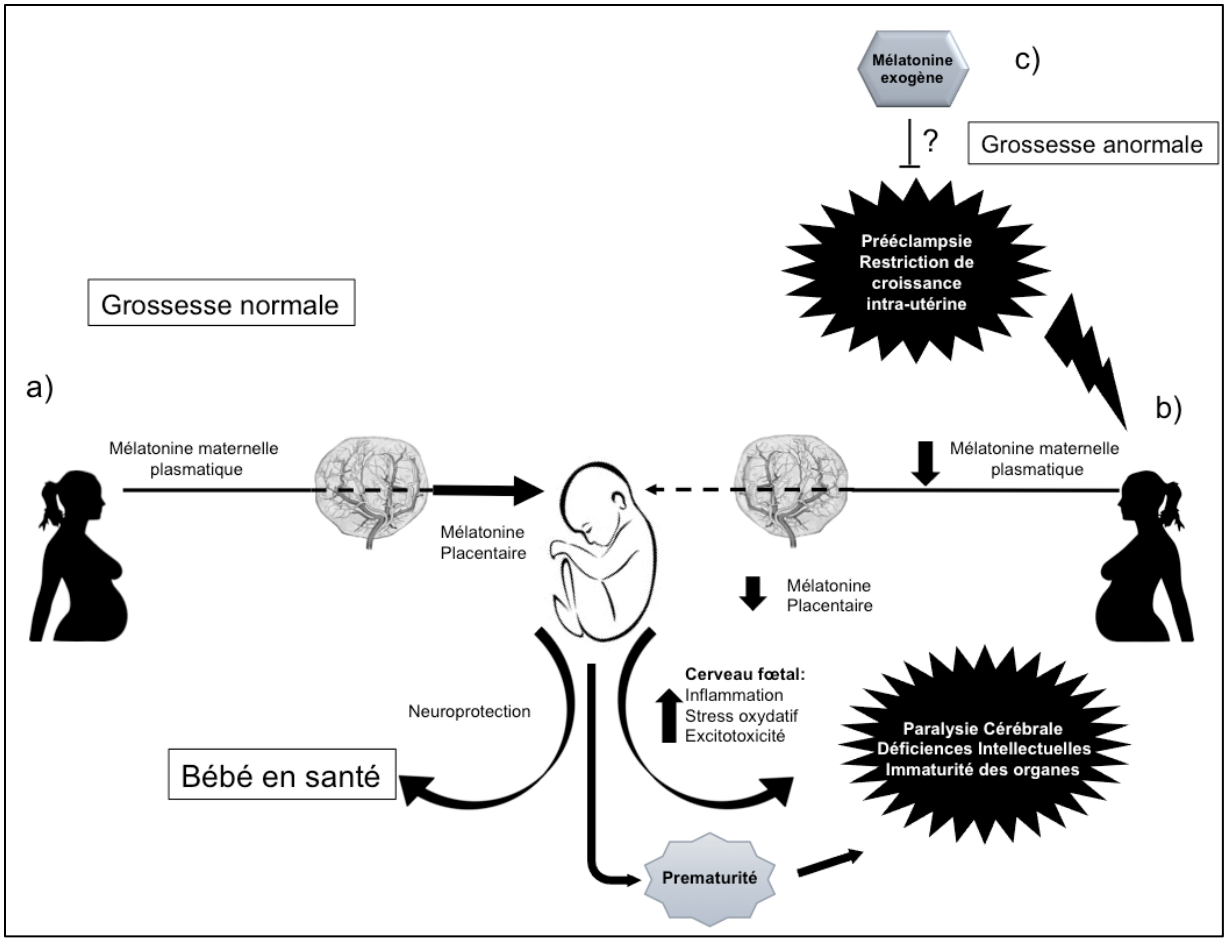

Figure 12: Effets de la mélatonine sur la grossesse et le développement du cerveau du foetus. A) Dans les grossesses normales, on observe une augmentation de la concentration de mélatonine plasmatique maternelle au cours de la grossesse. La mélatonine placentaire et maternelle atteint le cerveau du fœtus et, via des récepteurs spécifiques ou directement avec des actions antioxydantes, protège et module la réplication et la différenciation neuronale. B) Les maladies de la grossesse, tels que la prééclampsie et la restriction de croissance intra-utérine sont associées à une augmentation du stress oxydatif, qui affecte négativement l'ensemble de l'unité mère-placenta-fœtus, entraînant une augmentation du stress oxydatif, de l'inflammation et de l'excitotoxicité dans le cerveau du fœtus et la prématurité, avec des effets neurologiques à long terme. Ces maladies de grossesse sont corrélées avec des taux plasmatiques maternels et placentaires de la mélatonine sont diminués. C) La mélatonine exogène pourrait être utilisée comme traitement contre les maladies de la grossesse et pour protéger le cerveau du fœtus (voir Annexe 3 (Sagrillo-Fagundes *et al*. 2016)).

1.5. Hypothèses et objectifs

1.5.1. Problématique

Notre équipe a précédemment démontré que l'expression des enzymes de synthèse et des récepteurs de la mélatonine ainsi que la production de cette indolamine sont altérées dans les placentas de grossesses compliquées par une prééclampsie (Lanoix *et al*. 2008; Lanoix *et al.* 2012; Yamamoto *et al*. 2013). Plusieurs laboratoires, dont le nôtre, ont démontré que l'exposition de primoculture de vCTBs à une H/R mime les dommages cellulaires causés par la prééclampsie, soit l'activation de l'apoptose intrinsèque et une augmentation du stress oxydatif. Notre équipe a démontré que la mélatonine renverse ces dommages induits par l'H/R dans le vCTB *in vitro* (Lanoix *et al*. 2013). Ces travaux suggèrent donc que l'exposition *in vitro* à l'H/R de primoculture de vCTBs est un bon modèle pour étudier le rôle de la mélatonine et ses récepteurs dans le maintien de l'homéostasie du trophoblaste villeux et dans les physiopathogenèse de grossesses associées à une H/R, comme la prééclampsie.

L'autophagie est un mécanisme de protection activée pour dégrader les protéines et les organites endommagés par le stress oxydatif dans les cellules normales et dans les cellules tumorales (Wang *et al*. 2016). L'H/R est un activateur de l'autophagie, due à l'augmentation excessive des EROs principalement pendant la phase de réoxygénation, qui conduit également à une augmentation de l'inflammation et de l'apoptose. L'inflammation est un mécanisme physiologique actif au cours de la grossesse. Elle constitue une réponse protectrice impliquant les cellules immunitaires, et des médiateurs moléculaires pour garantir le bon développement foetoplacentaire. Par contre, dans certaines maladies de la grossesse, l'inflammation est exacerbée et a des effets néfastes sur le développement du fœtus et la santé de la mère (Kalkunte *et al*. 2011; Kim *et al*. 2015). Des études suggèrent que la mélatonine régule l'autophagie et l'inflammation afin de maintenir l'homéostasie des cellules normales en situation de stress. A contrario, dans les cellules tumorales, la mélatonine aurait un rôle cytotoxique et serait capable d'induire la mort cellulaire par l'inhibition des voies cellulaires protectrices, dont l'autophagie (Reiter *et al.* 2017). Par contre, le rôle de la mélatonine dans la régulation de l'autophagie et de l'inflammation trophoblastique en condition d'H/R n'a jamais été étudié. De même, la comparaison des actions de la mélatonine sur l'autophagie et l'inflammation des cellules trophoblastiques normales et des tumorales n'est pas connue.

1.5.2. Hypothèses

S'appuyant sur les travaux précédents de notre groupe et sur les données de la littérature, les hypothèses de recherche du présent projet doctoral sont :

1) La mélatonine régule l'autophagie et l'inflammation protégeant le trophoblaste villeux contre les dommages induits par une H/R ;
2) La mélatonine module l'autophagie pour maintenir la survie/l'homéostasie des cellules trophoblastiques normales et induire la mort des cellules tumorales.

1.5.3. Objectif général

L'objectif général est de déterminer les mécanismes cellulaires par lesquels la mélatonine régule l'autophagie et l'inflammation dans les trophoblastes placentaires humains.

1.5.4. Objectifs spécifiques

Les objectifs spécifiques sont :

1) Déterminer si et par quelles voies de signalisation la mélatonine régule l'inflammation et l'autophagie dans les primoculture de vCTBs et de STB cultivés en normoxie et H/R;

2) Déterminer si la mélatonine régule différemment l'autophagie dans les cellules trophoblastiques normales (primocultures) et tumorales (cellules BeWo).

2. ARTICLE : HUMAN PRIMARY TROPHOBLAST CELL CULTURE MODEL TO STUDY THE PROTECTIVE EFFECTS OF MELATONIN AGAINST HYPOXIA/REOXYGENATION-INDUCED DISRUPTION

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Contribution de l'étudiant

Lucas Sagrillo-Fagundes a participé à l'élaboration de l'étude, réalisé les expériences et analysé les résultats. Il a également rédigé l'article et participé au choix du journal de publication et effectué les corrections demandées par les évaluateurs.

L'article a été soumis à JOVE le 14 juin 2016 et accepté pour publication le 30 juillet 2016.

Résumé de l'article en français

Cette étude décrit l'isolation par des digestions enzymatiques successives, suivies par centrifugation sur gradient de densité, et immunopurification des cytotrophoblastes villeux (vCTB) humains de placentas humains de grossesses normales à terme. Comme observées *in vivo*, les cultures primaires de vCTB se différencient en syncytiotrophoblaste (STB) après 72 heures. Comparés à la normoxie (8% O_2), les vCTBs exposés à une hypoxie/réoxygénation (H/R) (0,5% - 8% O_2) subissent un stress oxydatif et une apoptose intrinsèque, similaires à celle *observée in vivo* dans les complications de la grossesse telles que la prééclampsie, la naissance prématurée et la restriction de la croissance intra-utérine. Dans ce contexte, les trophoblastes villeux primaires cultivés dans ces conditions d'H/R représentent un modèle *in vitro* unique pour mieux comprendre les mécanismes et les voies de signalisation qui sont modifiés dans les placentas in vivo et pour faciliter la recherche de médicaments efficaces contre les maladies de la grossesse. La mélatonine a été suggérée comme traitement pour la prééclampsie et la restriction de croissance intra-utérine en raison de ses effets antioxydants. Les trophoblastes villeux humains produisent de la mélatonine et expriment ses enzymes de synthèse et ses récepteurs. Dans le modèle de primoculture de vCTB décrit dans cet article, la mélatonine n'a aucun effet sur les cellules en condition normoxique, mais restaure l'équilibre redox perturbé par l'H/R. Cette étude suggère que les primocultures de trophoblastes villeux humains sont un excellent modèle pour étudier les effets protecteurs de la mélatonine sur les fonctions placentaires altérées en situation d'H/R.

Video Article

Human Primary Trophoblast Cell Culture Model to Study the Protective Effects of Melatonin Against Hypoxia/reoxygenation-induced Disruption

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URL: http://www.jove.com/video/54228
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Abstract

This protocol describes how villous cytotrophoblast cells are isolated from placentas at term by successive enzymatic digestions, followed by density centrifugation, media gradient isolation and immunomagnetic purification. As observed *in vivo*, mononucleated villous cytotrophoblast cells in primary culture differentiate into multinucleated syncytiotrophoblast cells after 72 hr. Compared to normoxia (8% O_2), villous cytotrophoblast cells that undergo hypoxia/reoxygenation (0.5% / 8% O_2) undergo increased oxidative stress and intrinsic apoptosis, similar to that observed *in vivo* in pregnancy complications such as preeclampsia, preterm birth, and intrauterine growth restriction. In this context, primary villous trophoblasts cultured under hypoxia/reoxygenation conditions represent a unique experimental system to better understand the mechanisms and signalling pathways that are altered in human placenta and facilitate the search for effective drugs that protect against certain pregnancy disorders. Human villous trophoblasts produce melatonin and express its synthesizing enzymes and receptors. Melatonin has been suggested as a treatment for preeclampsia and intrauterine growth restriction because of its protective antioxidant effects. In the primary villous cytotrophoblast cell model described in this paper, melatonin has no effect on trophoblast cells in normoxic state but restores the redox balance of syncytiotrophoblast cells disrupted by hypoxia/reoxygenation. Thus, human villous trophoblast cells in primary culture are an excellent approach to study the mechanisms behind the protective effects of melatonin on placental function during hypoxia/reoxygenation.

Video Link

The video component of this article can be found at http://www.jove.com/video/54228/

Introduction

Throughout human pregnancy, the placental cytotrophoblast cells, which are mononucleated stem cells, rapidly proliferate and differentiate into either villous or extravillous cytotrophoblast cells. Extravillous cytotrophoblasts invade and remodel the spiral arteries of the uterine wall. Villous cytotrophoblasts, on the other hand, continue to proliferate, differentiate and fuse to form multinucleated syncytiotrophoblast (the syncytium)[1]. The maintenance of villous trophoblast homeostasis is essential for fetal well-being and healthy pregnancy. In fact, villous trophoblasts allow maternal-fetal exchange of oxygen and nutrients, and produce essential hormones for pregnancy. Moreover, the syncytiotrophoblast is the only cell-type in direct contact with the maternal blood circulation and provides an essential physical and immunological barrier. Therefore, the syncytiotrophoblast must undergo apoptosis and replacement for homeostatic maintenance and to avoid placental pathologies[2-5].

The technique developed by Kliman *et al.*[6] in 1986 to isolate primary villous cytotrophoblasts from human placentas caused a revolution in placental research by allowing the study of the molecular mechanisms involved in villous trophoblast differentiation. This classical technique, based on sequential enzymatic digestions with trypsin and DNase, followed by isolation in density centrifugation media (colloidal silica particles coated by polyvinylpyrrolidone, or Percoll) is now recognized as the gold standard for isolating villous cytotrophoblast cells. The technique can be optimized by magnetic immunopurification, a procedure that separates villous cytotrophoblasts from non-trophoblastic cells based on the differential expression of specific antigens on the surfaces of these cells. We chose the human leukocyte antigen ABC (HLA-ABC) due to the absence of its expression on the trophoblastic cell membrane[7,8].

The placenta is an organ that undergoes dramatic variations in oxygen levels during pregnancy. In the first trimester, the oxygenation ratio is physiologically very low (2% O_2) but increases to mild levels of oxygenation (8% O_2) in the second and third trimester. Tuuli *et al.*[9] described that the *in vitro* reproduction of the trophoblast environment inside the placental villi is a challenge and variations in oxygenation levels may even lead to phenotypical changes. It is, therefore, suggested to adopt 8% oxygen as normoxia to mimic the oxygen tension found in placental villi during the third trimester of gestation[8,9]. Chen *et al.*[10] extensively studied several variables related to oxygen tension in trophoblast cell culture and demonstrated the importance of determining oxygen levels in a pericellular environment. The levels of oxygen in the villi tend to increase

due to vasculogenesis. The blood flow in placental villi increases constantly and the level of hydrogen peroxide (an abundant reactive oxygen species) is an important signal that controls vasculogenesis[11,12]. In pregnancy complications, a lack of vasculogenesis generates hypoxia, and more importantly, intermittent variations of oxygenation (called hypoxia/reoxygenation). These conditions lead to an abnormal increase in oxidative stress, which compromises placental and fetal viability[13,14]. The alterations that trophoblast cells undergo *in vivo* during episodes of hypoxia/reoxygenation can be mimicked *in vitro* as follows: villous cytotrophoblasts are maintained under normoxic conditions (8% O_2) until they differentiate into syncytiotrophoblast. They are then subjected to hypoxic conditions (0.5% O_2) for 4 hr, followed by an additional 18 hr of normoxia (reoxygenation). Using this hypoxia/reoxygenation approach, trophoblasts exhibit deregulated redox status and increased levels of intrinsic apoptosis[8], as has been observed in certain pregnancy complications. Hence, this is a useful *in vitro* model to evaluate new preventive and therapeutic approaches to combat pregnancy complications associated with placental hypoxia/reoxygenation.

Placental cells produce melatonin, which has several important functions, such as an ability to obviate oxidative stress and placental dysfunction[15]. Here, we present the experimental approach and cell models used to demonstrate the protective effects of melatonin in placental trophoblast cells at the molecular, cellular and functional level[8].

Protocol

Placentas were obtained immediately after spontaneous vaginal deliveries from uncomplicated pregnancies at the CHUM-St-Luc Hospital, Montreal, QC, Canada, with informed patient consent and approval of ethical committees (CHUM-St-Luc Hospital and INRS-Institut Armand-Frappier, Laval, QC, Canada).

1. Isolation and Purification of Villous Cytotrophoblast Cells

1. Solutions and media
    1. Prepare transport media by supplementing Dulbecco's Modified Eagle's Medium High-Glucose (DMEM-HG) with 1% *vol/vol* antibiotic (10,000 units/ml penicillin G, 100 mg/ml streptomycin sulphate) and store at 4 °C.
    2. Prepare primary culture media by supplementing DMEM-HG with 10% *vol/vol* fetal bovine serum (FBS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% *vol/vol* antibiotic (10,000 units/ml penicillin G, 100 mg/ml streptomycin sulphate) and store at 4 °C. Warm media to 37 °C before use.
    3. Prepare 4 L of saline solution (0.9% weight/vol sodium chloride).
    4. Prepare modified Hank's Balanced Salt Solution (HBSS) by adding 25 mM HEPES to 1x HBSS (pH 7.4).
    5. Prepare fresh, four bottles of digestion solution with modified HBSS (prepared in 1.1.4), magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), trypsin, deoxyribonuclease IV (DNase IV), and 1% *vol/vol* antibiotic (10,000 units/ml penicillin G, 100 mg/ml streptomycin sulfate) as shown in **Table 1**.
    6. Prepare density centrifugation media gradient
        1. Prepare density centrifugation media solution by supplementing density centrifugation media with 10% *vol/vol* HBSS 10x.
        2. Prepare 14 assay tubes with density centrifugation media solution and modified HBSS, as described in **Table 2**.
        3. Mix each density solution (step 1.1.6.2) vigorously before adding its content to the gradient. Gently add the density solutions to a 50 ml glass centrifuge tube with a peristaltic pump (1 ml/min), beginning with the highest concentration (70%). Avoid droplets by draining the solutions against the tube wall to maintain the proper separation of each layer of the gradient.
            1. In absence of a peristaltic pump, apply the layers very gently with Pasteur pipettes.
    7. Prepare final running buffer by supplementing the running buffer (see Table of Materials) with 2% *vol/vol* antibiotic/antimycotic (10,000 units/ml penicillin G, 100 mg/ml streptomycin sulfate). Store at 4 °C.

2. Villous cytotrophoblast isolation
    Note: Use sterile surgical equipment, glassware, pipettes, flasks, *etc.*
    1. On the day of villous cytotrophoblast isolation, in a 37 °C water bath, warm the digestions solutions (from step 1.1.5) and 70 ml of FBS. Note: Use 50 ml of FBS to interrupt the digestions and the remaining 20 ml for the freezing step (1.2.22) which can be placed on ice once thawed.
    2. After delivery, bring the placenta to the laboratory in ice-cold transport medium (from step 1.1.1) as quickly as possible (less than 1 hr).
    3. Discard transport medium and placental blood in liquid dustbin. Weigh the placenta and immerse in cold saline solution.
    4. Measure and analyze the following features: umbilical cord length; umbilical cord localization; placental length, width, shape (oval, discoid); membrane color; cotyledon structure pathologies. Note: Data are presented in the results section.
    5. Cut the umbilical cord alongside its placental insertion (*i.e.*, at its base with an additional 1 cm radius circle around the cord). Immerse in 300 ml of histological tissue fixative solution (formalin 10%) for later histological analysis.
    6. Cut the entire placenta into cubes of 5 x 5 x 5 cm. Wash thoroughly (4 times x ~ 1 min) in saline solution (0.9%) to remove blood cells until saline solution is clear. Discard rinsing liquid.
    7. In a watch glass, remove placental membranes and mince tissues to remove blood vessels and calcifications. Hold blood vessels firmly with forceps and remove tissues using the back of Metzenbaum scissors.
        1. Place minced placenta in a Büchner funnel. Rinse with approximately 100 ml of saline buffer. Continue mincing until 30 - 35 g of minced tissue is obtained (use plastic weighing boat and scale). If needed, mince the remainder of the placenta to obtain up to three additional 30 - 35 g preparations. During this time, put minced tissue in a weighing boat on ice.
        Note: This step will take around 45 min to 1 hr.
    8. Add the 30 - 35 g minced placental tissue to a trypsinizing flask. Transfer 150 ml of the prepared digestion solution 1 (**Table 1**) to the trypsinizing flask and mix well.
    9. Place the trypsinizing flask in a shaking water bath for 30 min at a speed of no more than 50 cycles/min and manually mix the trypsinizing flask every 5 min for homogenous digestion.

10. At the end of the first digestion, remove the trypsinizing flask from the water bath and tilt it (45°) for 1 min to sediment the placental tissue. With a 10 ml sterile pipette, remove and discard approximately 80 ml of supernatant. Avoid aspirating the tissue.
11. Transfer 100 ml of digestion solution 2 (**Table 1**) to the trypsinizing flask; mix well and repeat step 1.2.9.
12. At the end of the second digestion, remove the trypsinizing flask from the water bath and tilt it 45° for 1 min. With a 10 ml sterile pipette, remove 80 ml supernatant and gently transfer to a centrifuge tube with a cell strainer (100 µm mesh). Transfer the filtered supernatant to a beaker containing 2 ml of FBS every time the centrifuge tube is full.
13. Perform the third digestion as described for the second digestion (1.2.11 and 1.2.12) using 75 ml digestion solution 3. In parallel, perform steps 1.2.15 to 1.2.16.1 for digestion 2.
14. Perform the fourth digestion exactly as the third digestion using the digestion solution 4 (75 ml), but collect a maximum amount of supernatant. Perform steps 1.2.15 to 1.2.16.1 in parallel for digestion 3, and finally for digestion 4.
15. Aliquot the supernatant (from digestions 2, 3 and 4) into 13.5 ml parts, each part into one 15 ml centrifuge tube. With a 22.8 cm long glass Pasteur pipette, very gently and slowly place 1.5 ml of FBS at the bottom of each tube in order to create a separate layer. Do not mix FBS and supernatant. Centrifuge the tubes without brake for 20 min at 1,250 x g at room temperature.
    Note: After centrifugation, 4 layers are visible in the tube, as shown in **Figure 1**. The separation of trypsin and trophoblast cells avoids excessive cellular digestion.
16. With a vacuum pump, aspirate and discard the supernatant (digestion solution) and FBS layers, including the whitish film between the two layers. Resuspend the pellet (the trophoblasts and red blood cell layers) with 1 ml of warm cell culture medium (step 1.1.2; with no FBS and no HEPES).
    1. Collect resuspended cells from all tubes and combine them in 1 tube. Let the tube stand at room temperature until the end of all digestions. Note: After all digestions, the usual yield is 3 tubes of resuspended cells (one per digestion).
17. Make up the volume to 15 ml with warm cell culture medium. Centrifuge at 1,250 x g for 10 min at room temperature. Remove the supernatant with a vacuum pump. Avoid aspirating the pellet.
18. Gently resuspend the pellet obtained from the 3 tubes with 1 ml of warm cell culture medium. Pool their content in 1 tube. To obtain 8 ml, complete the volume with warm cell culture medium.
19. Very gently layer the cell suspension on a separation gradient with a Pasteur pipette. Centrifuge without brake for 30 min at 507 x g at room temperature.
20. After centrifugation, identify the different layers of cells in the gradient with back-lighting. Locate the layers containing trophoblast and contaminating cells between 40 - 50% of density centrifugation medium. With a vacuum pump, remove upper layers (> 50%).
21. Collect cells located in the layers of interest with a Pasteur pipette and transfer them to a 50 ml centrifuge tube. Make up the volume to 50 ml with cell culture medium. Centrifuge for 10 min at 1,250 x g at room temperature.
22. Under sterile conditions, discard the supernatant, resuspend the pellet with 20 ml of FBS and count the number of cells using a hemocytometer. On ice, add 2.22 ml of sterile dimethyl sulfoxide (DMSO) and mix gently by flipping. Aliquot 1.5 ml of cell suspension into cryogenic vials, freeze overnight at -80 °C and transfer to a liquid nitrogen tank.

3. Trophoblast purification
    1. Install the rinsing and running buffers and a new filter column on the magnetic purification instrument according to manufacturer's instructions. Perform the "clean program" to clean negative 1, positive 1 and positive 2 ports, and then introduce the 50 ml tubes under each port according to manufacturer's instructions.
    2. Thaw the cells that were frozen in step 1.2.22 quickly in a 37 °C water bath. Transfer cells to a 50 ml tube and resuspend cells gently with 20 ml of cold running buffer solution. Centrifuge the tube for 5 min at 450 x g and 4 °C.
    3. Discard the supernatant. Repeat the wash step with cold running buffer. Count cells using a hemocytometer to determine viability. Repeat the centrifugation (for 5 min, 450 x g at 4 °C). Carefully remove the supernatant. Add 1 ml of cold running buffer containing 1% *vol/vol* of mouse anti-HLA-ABC antibodies. Incubate at 4 °C for 30 min, mixing gently every 5 min.
    4. Add 6 ml of cold running buffer. Centrifuge for 5 min at 450 x g and 4 °C. Discard the supernatant and repeat this step. Resuspend cells in 1 ml of cold running buffer containing 10% *vol/vol* of anti-mouse secondary antibody-coupled magnetic beads. Incubate at 4 °C for 15 min, mixing gently every 5 min.
    5. Add 6 ml of cold running buffer. Centrifuge for 5 min at 450 x g and 4 °C. Discard the supernatant and resuspend in 5 ml cold running buffer.
    6. Separate the trophoblast cells using the magnetic purification instrument. Collect cells at the negative port and add 20 ml of cold running buffer.
        Note: Trophoblast cells do not contain the complex HLA-ABC, and are thus separated from other cell types and directed towards the negative 1 port.
    7. Centrifuge for 5 min at 450 x g and 4 °C. Discard the supernatant and gently resuspend the cells in 20 ml warm primary culture medium. Count the cells using a hemocytometer to determine viability.
    8. Plate the cells at the following densities: 0.15 x 10^6 cells/well in 96-well plates, 1.6 x 10^6 cells/well in 24-well plates and 4.5 x 10^6 cells/well in 6-well plates. Incubate plates at 37 °C and 5% CO_2.
    9. Confirm the purity of the trophoblast cells by flow cytometry[16,17] and/or by immunocytochemistry[18].
        Note: The purity of the immunopurified cells was determined using FITC-conjugated monoclonal antibodies against cytokeratin-7 and vimentin[17-19]. This protocol is well detailed in Lanoix *et al.*, 2008[7].
    10. After at least 4 hr, rinse the cells twice with warm culture medium to remove unattached cells and then transfer the plates to the normoxia chamber, which is composed of 8% O_2 (see **Figure 2** and section 2).

2. *In Vitro* Induction of Normoxia and Hypoxia/Reoxygenation

1. Incubator chamber operation (see **Figure 2A** for set-up).
    1. In a laminar flow hood, place a Petri dish containing sterile water at the bottom of the incubator chamber to avoid dryness; then place the previously prepared cell culture plates or flasks (step 1.3.10) on the superior shelves of the chamber.

2. Outside the hood, attach the chamber (inlet port) to the gas hose (**Figure 2A**: 5a, b and c) to reach the tube of gas (8% O_2 or 0.5% O_2) (**Figure 2A**: 7). Open both inlet and outlet ports of the chamber. At this moment, the gas regulator (**Figure 2A**: 4) should remain closed.
3. Carefully open the gas regulator valve (**Figure 2A**: 4). Flush for 4 min with an air flow of 25 L/min to completely replace the air inside the chamber.
4. After flushing the chamber, close the gas regulator then the inlet and outlet ports of the chamber.
5. Unplug the flow meter outlet hose (**Figure 2A**: 5c) from the inlet port of the chamber and place the chamber in a cell culture incubator at 37 °C.
6. Replace the air currently present in the plates, flasks and dissolved in the culture medium by filling the chamber with gas 1 hr after step 2.1.3.
7. Repeat steps 2.1.1 to 2.1.6 for the other gas compositions (*e.g.*, 2% O_2 for first trimester trophoblast culture[9]).

2. Confirm the oxygen percentage (**Figure 2B-C**)
   1. To confirm the concentration of oxygen in the cell culture medium (without cells) inside the chamber, use an oxygen electrode connected to an oxygen adapter. Connect the oxygen electrode to a voltmeter.
   2. Create a calibration curve in the same solution (*i.e.*, cell culture medium) by exposing the solution to gases with known oxygen contents (*e.g.*, 0% and 21% oxygen). After the readings are stabilized for each concentration, introduce the electrode into the medium in the chamber.
      Note: Take all measurement at the same depth in order to avoid any bias in oxygen concentration[10].

3. Induction of normoxia and hypoxia/reoxygenation in trophoblasts.
   1. After adding primary trophoblast cells to the appropriate cell culture flasks, plates or Petri dishes, perform treatments as necessary.
   2. In parallel, inside the chamber, expose cells to the desired gas mixture to reproduce a specific condition every 24 hr (**Figure 3**).

Representative Results

Isolation and immunopurification of villous cytotrophoblast cells from a normal term placenta obtained by vaginal delivery yielded 1 x 10^8 viable cells. The placenta weighed 350 g, was 19 cm in diameter, 4 cm tall with discoid shape and transparent membranes. No cotyledon malformation was detected. The umbilical cord had paracentral localization and a length of 56 cm. The purity was evaluated by flow cytometry using vimentin and cytokeratin-7 markers. More than 98% of the cells were negative for vimentin and positive for cytokeratin-7, confirming the purity of villous trophoblasts cells obtained from the immunopurification. Villous cytotrophoblast cells were added to 96-well culture plates under normoxic conditions in the presence or absence of 1 mM melatonin. The biochemical differentiation of villous cytotrophoblasts was monitored by determining levels of β-human chorionic gonadotropin (β-hCG) secretion as described previously[1,7,20,21]. The morphological differentiation and apoptosis were assessed by immunofluorescence using anti-desmoplakin and anti-caspase-cleaved cytokeratin 18 intermediate filaments[7,22]. Cell culture media from day 1 (mainly villous cytotrophoblasts) to day 4 (mainly syncytiotrophoblasts) were collected, centrifuged and β-hCG levels were measured in the supernatants. Production of β-hCG, which is exclusive to the syncytiotrophoblast, increased with culture time (**Figure 4**). Not only hypoxia/reoxygenation, but hyperoxia (> 20% O_2) also activated apoptosis[23]. Thus, adoption of an 8% O_2 concentration was representative of the quantity of oxygen to which a villous trophoblast cell would be exposed during the third trimester of pregnancy[10]. The peak of β-hCG levels observed at 72 hr confirmed the capacity of villous cytotrophoblasts to differentiate under these conditions. Melatonin did not alter β-hCG secretion under these study conditions. The decrease of β-hCG levels at 96 hr was likely caused by apoptosis of trophoblast cells, which increases after prolonged periods in primary culture[5,7,22,24,25] (**Figure 4**). DMSO (0.1% *vol/vol*) was selected because it did not affect β-hCG levels[26,27]. The protective role of melatonin was strongly related to its antioxidant properties. Hypoxia/reoxygenation after 72h of culture induced oxidative stress in villous trophoblast cells. The protective effect of melatonin was assessed with Reactive Oxygen Species (ROS) Detection Reagent (**Figure 5A**). After 96 hr of culture, trophoblast cells were incubated for 45 min with 10 µM of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) to detect the total amount of ROS produced[8]. Villous trophoblast cells that underwent hypoxia/reoxygenation had significantly increased ROS levels (54%) compared to those under normoxia. This increase was reversed by treatment with 1 mM melatonin. Moreover, under normoxia melatonin did not modulate ROS levels (homeostasis), which was similar to non-treated villous trophoblast cells (**Figure 5A**). **Figure 4** and **5A** show that under normoxia melatonin did not alter levels of oxidative stress or β-hCG secretion in the trophoblast cells, which corroborates previous studies showing no modulation of cell homeostasis under normal conditions[28,29].

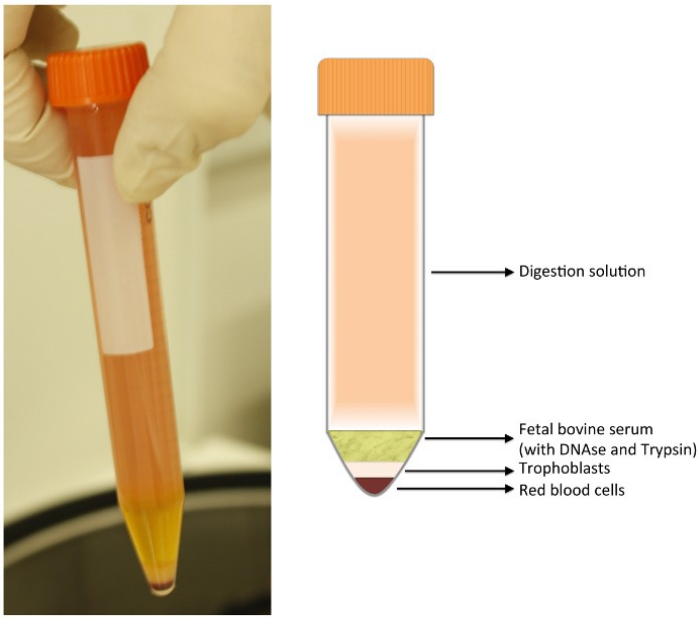

Figure 1: Digestion Tube. After centrifugation, 4 layers are formed. The upper layer is composed of digestion solution; just below, the fetal bovine serum (FBS). Both layers should be discarded with a vacuum pump. The lower layers are composed as follow: a white layer containing fibroblasts, leukocytes, macrophages, and trophoblasts; and a bottom layer composed of red blood cells. Please click here to view a larger version of this figure.

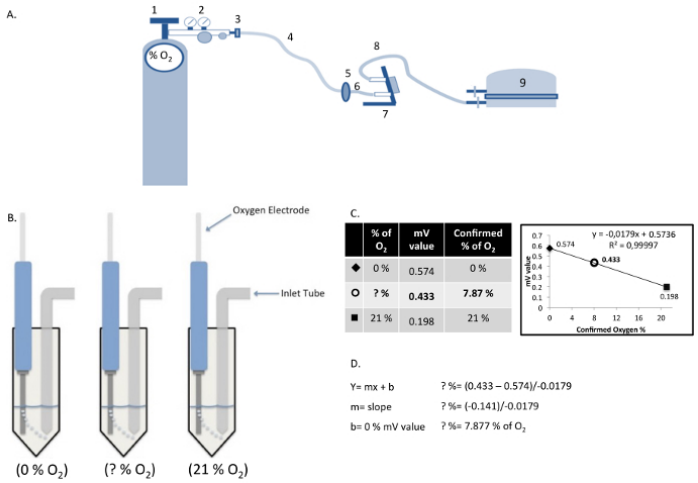

Figure 2: Components of the Hypoxia Chamber and Measurement/Calculation of Dissolved Oxygen Concentration. (A) Hypoxia chamber and gas cylinder assembly: (1) Gas cylinder; (2) Gas regulator; (3) Gas hose clamp; (4) Cylinder gas hose; (5) Inlet filter; (6) Inlet hose; (7) Flow meter; (8) Outlet hose; (9) Modular incubator chamber. **(B)** Calculation of actual oxygen concentration in cell culture medium using a standard curve produced with known oxygen concentrations. (**C and D**) The relative values obtained in the solutions "0% O_2" and "21% O_2", are plotted graphically as a linear function to determine the oxygen concentration in the cell culture medium "?% O_2". Please click here to view a larger version of this figure.

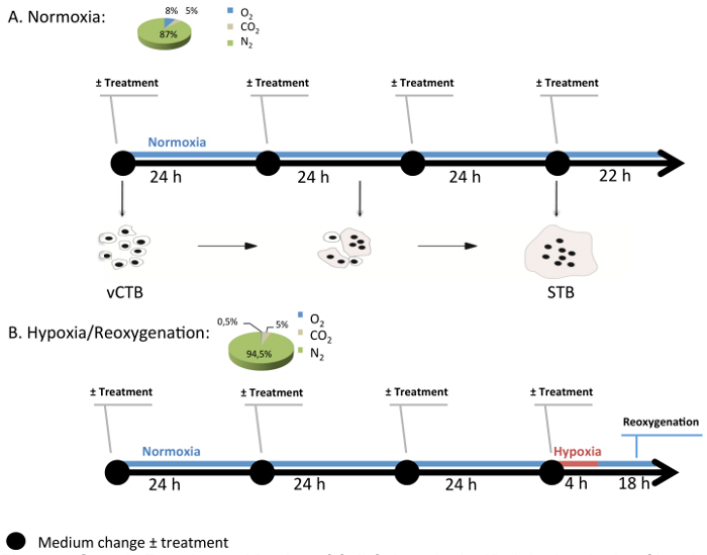

Figure 3: Generic Experimental Design of Cell Culture in the Modular Incubation Chamber. Normoxia (8% O_2; 5% CO_2; 87% N_2) and hypoxia/reoxygenation (H/R) (0.5% O_2; 5% CO_2; 94.5% N_2) are conditions used to study pathological conditions in villous cytotrophoblast (vCTB) and syncytiotrophoblast (STB) cells. Every 24 hr, medium with or without melatonin (1 mM) is changed and the gas mixture is renewed. Under H/R, STB cells undergo hypoxia (0.5% O_2) for 4 hr and then return to normoxia (8% O_2). Please click here to view a larger version of this figure.

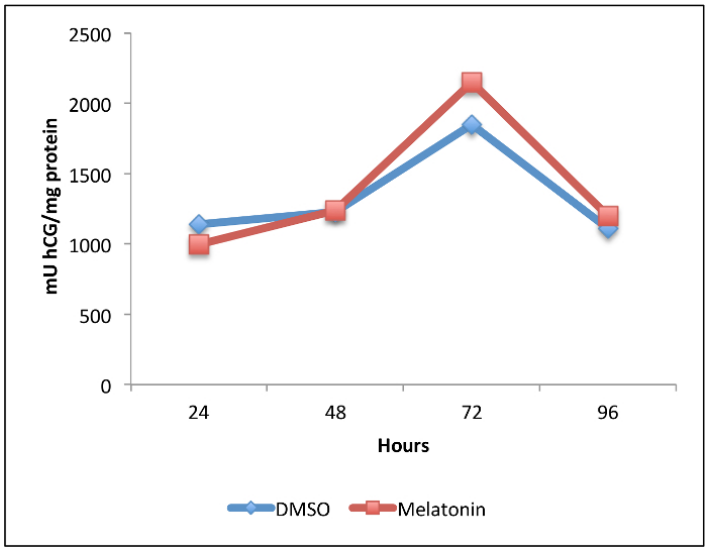

Figure 4: Effect of Melatonin on beta-human Chorionic Gonadotropin (β-hCG) Secretion during Villous Trophoblast Differentiation. Villous cytotrophoblast cells were isolated and purified from human healthy term placentas. Cells were treated for 96 hr with 1 mM melatonin or dimethyl sulfoxide (DMSO 0.1%: vehicle control) under normoxic conditions (8% O_2; 5% CO_2; 87% N_2). β-hCG levels in culture medium were measured by enzyme-linked immunosorbent assay (ELISA) after 24, 48, 72 and 96 hr of primary culture. Levels were normalized to the protein content of the whole-cell lysate from each corresponding well. Please click here to view a larger version of this figure.

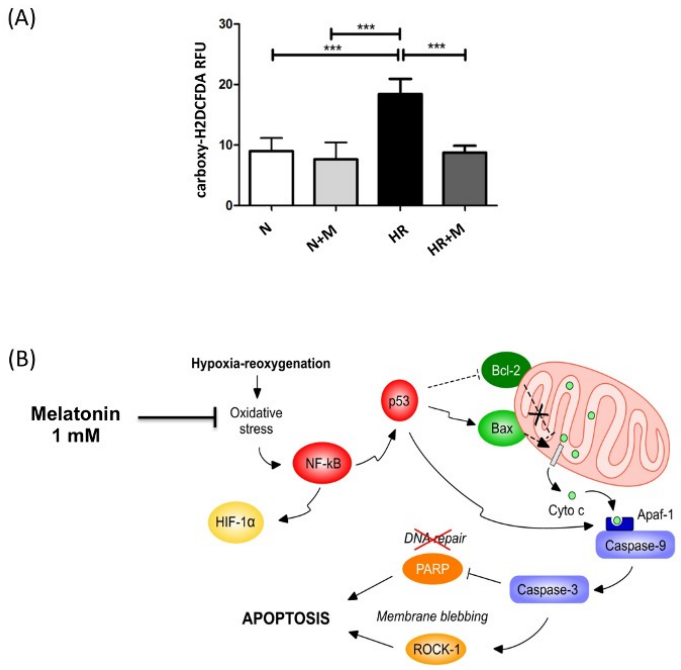

Figure 5: Anti-oxidant Effect of Melatonin in Syncytiotrophoblast Exposed to Hypoxia/Reoxygenation. **(A)** The effect of melatonin (M; 1 mM) on intracellular reactive oxygen species (ROS) levels in syncytiotrophoblast cells under normoxia (N) or hypoxia/reoxygenation (HR), induced after 72 hr of culture, was assessed by 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) fluorescence. Results are expressed as the mean ± SD of 3 different placentas; *** $P < 0.001$ (Lanoix, *et al.*[8]). **(B)** The cellular pathways involved in the putative protection of melatonin against hypoxia/reoxygenation-induced apoptosis. Primary villous cytotrophoblast cells were cultured for 72 hr under normoxia (8% O_2) to allow differentiation into syncytiotrophoblast. Cells were exposed to 1 mM of melatonin or vehicle control and then subjected to hypoxia (0.5% O_2) for 4 hr followed by an 18 hr reoxygenation period (8% O_2). Hypoxia/reoxygenation-induced oxidative stress activates redox sensitive transcription factors such as nuclear factor kappa B (NF-κB) and hypoxia inducible factor 1 (HIF-1). NF-κB induces p53, which triggers the Bax/Bcl-2 pathway of mitochondrial apoptosis involving the cleavage and activation of caspases 9 and 3. Caspase 3 activates Rho-associated, coiled coil-containing protein kinase 1 (ROCK-1), the cleavage of poly(ADP-ribose) polymerase (PARP) and the impairment of DNA repair. Melatonin prevents the induction of mitochondrial apoptosis by acting as a powerful antioxidant to reduce the oxidative stress caused by hypoxia/reoxygenation. This figure has been modified from Lanoix *et al.*, 2013[8]. Please click here to view a larger version of this figure.

	Digestion 1	Digestion 2	Digestion 3	Digestion 4
Modified HBSS (ml)	150	100	75	75
DNAse (µl)	300	200	150	150
(0.1 mg/µl)				
$MgSO_4$ (µl)	150	100	75	75
(800 mM)				
$CaCl_2$ (µl)	150	100	75	75
(100 mM)				
Trypsin (U)	1,824,000	1,200,000	960,000	960,000
P/S (ml)	1	0	0	0

Table 1: Quantities of Ingredients for the Digestion Solution. Penicillin and streptomycin (P/S); magnesium sulfate ($MgSO_4$); calcium chloride ($CaCl_2$); deoxyribonuclease IV (DNase IV).

Gradient Concentration (%)	70	65	60	55	50	45	40	35	30	25	20	15	10	5
Density centrifugation media solution (ml)	2.33	2.17	2	1.83	1.67	1.5	1.33	1.17	1	0.83	0.67	0.5	0.33	0.17
Modified HBSS (ml)	0.67	0.83	1	1.17	1.33	1.5	1.67	1.83	2	2.17	2.33	2.5	2.67	2.83

Table 2: Volumes of Density Centrifugation Media Solution and Modified HBSS Required for Preparation of the Gradient Solution.

Discussion

In mammals, fetal development is directly dependent on adequate placental function. The developmental origins of health disorders are based on the hypothesis that the cause of diseases manifested later in life can be traced back to early development and that the placenta has a mechanistic role in fetal programming[30-32]. The placenta is the key mediator of fetal growth and development: it regulates nutrient transfer, protects against harmful exposures, and has major endocrine functions. The development by Kliman ***et al.*** of a reproducible technique to isolate viable primary cytotrophoblasts is a milestone in the study of normal and abnormal placental functions[6]. Many researchers have adapted this technique to reproduce specific conditions *in vitro* to understand placental physiology[18,20,33,34]. As described by Petroff *et al.*, many steps are important to guarantee a purified and robust yield of isolated cytotrophoblast cells[18]. For example, the digestion steps have undergone several modifications since the development of the technique in 1988, such as an increased number and length of the digestions, as well as to the composition and quantity of digestion enzymes, resulting in greater numbers of viable isolated cytotrophoblasts[18]. This current protocol has three main advantages: cryopreservation, which allows for the possibility to continue the protocol later immunomagnetic purification, which increases cytotrophoblast purity and the use of pre-coated microplates improving cell attachment[7,34-36]. The existing literature contains several examples of diverse modifications of the isolation technique for cytotrophoblast cells, but the characteristics of the density centrifugation media has remained virtually unmodified[37]. The presented technique of isolation/immunopurification has several critical steps. Hence, it is important to evaluate the purity of the villous cytotrophoblast cells at the end of each immunopurification. This can be done by flow cytometry using appropriate antibodies as markers: cytokeratin-7 (trophoblastic marker), cluster of differentiation 45 (CD45) and vimentin (non-trophoblast cells markers)[18,38,39]. Other critical aspects are the quality of the obtained placentas, the FBS and density centrifugation media gradient, as well as centrifugation speed, which can all influence the yield and quality of the cells[20,40].

Although widely used, the present technique has unavoidable limitations. Firstly, the amount of viable cytotrophoblasts cells obtained after immunopurification is relatively low and is the main limiting factor in the number of possible conditions/treatments that can be tested. Secondly, the life-span of primary trophoblast cells is short and *in vitro* differentiation into syncytiotrophoblast is closely followed by a reduction of cell viability and an increase of apoptosis. The short length of trophoblast cell viability which does not proliferate *in vitro,* limits the evaluation of longer term treatments, because apoptosis is irreversibly triggered after about 4 days of culture[7,41]. Thirdly, interplacental variability is large, so a relatively large number of placentas is required to obtain statistically interpretable results. On the other hand, primary trophoblast culture has unique advantages, such as the capacity of the cells to differentiate into syncytium, which allows for the study of conditions and treatments in different cell phenotypes according to the various stages of differentiation. The oxygenation method presented in this protocol is highly adaptable and its configuration can be tailored for other situations, such as the culture of primary trophoblast cells from first trimester pregnancy, which should be exposed to a lower oxygen tension for normoxia[9,42,43].

There are other approaches to study human placental function *in vitro*. Snap-freezing placental tissues allows for multi-omics analyses, but requires placental multisite sampling, to avoid for example metabolic variations due to the oxygenation gradient, which decreases from the central villi to the periphery[44]. However, live trophoblast cell biology and behavior cannot be studied using this approach[45]. Villous explants have the advantage of maintaining the whole villous structure with the constituent cell types and their communication, but responses to treatments are not specific to trophoblast cells[23]. Commercially available trophoblast-like choriocarcinoma cell lines, such as BeWo, Jeg-3 and JAR can be used to study placental functions, such as fusion, differentiation, and transplacental transport. However, recent studies show that gene expression in primary cytotrophoblasts and BeWo tumor cells are poorly correlated[46-48]. Thus, primary villous trophoblast cell culture, despite its limitations, possess the unique advantage of mimicking the *in vivo* environment of the normal or abnormal placenta.

Studies using villous trophoblast cell in primary culture and villous explants show that hypoxia and hypoxia/reoxygenation systematically decrease trophoblast cell viability, concomitant with increased levels of oxidative stress, inflammation, autophagy and apoptosis[43,49-52]. This hypoxia/reoxygenation cell culture model, specifically, has allowed us to demonstrate the antioxidant and anti-apoptotic effects of melatonin in villous trophoblast cells. In primary villous trophoblast cells exposed to hypoxia/reoxygenation, melatonin prevents the following: induction of oxidative stress, decreased antioxidant enzyme activities, increased activity of redox-sensitive signaling pathways, and induction of mitochondrial apoptosis (**Figure 5B**)[8]. The hypoxia/reoxygenation model is a unique tool to ascertain the preventive role of melatonin in oxidative stress-induced damage and its possible protective role in pregnancy complications such as preeclampsia, where placental melatonin synthesis is reduced[53]. Melatonin is a powerful antioxidant with a wide range of targets[54]. Also, the safety of melatonin as a treatment has been largely established. The beneficial results with melatonin have been reproduced by several researchers and melatonin is currently in clinical trials as a potential preventive or therapeutic treatment in pregnancies complicated by preeclampsia or intrauterine growth restriction[55,56].

In conclusion, the isolation, purification and primary culture of high quality cytotrophoblast cells, together with the technique of hypoxia/reoxygenation enable a wide range of promising experimental approaches to better understand pregnancy complications related to oxidative stress and improve placental health.

Disclosures

The authors have nothing to disclose.

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3. ARTICLE : INFLAMMATION AND AUTOPHAGY ARE MELATONIN TARGETS IN HUMAN PRIMARY TROPHOBLASTIC CELLS

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Contribution de l'étudiant

Lucas Sagrillo-Fagundes a participé à l'élaboration de l'étude, réalisé toutes les expériences et analysé les résultats. Il a également rédigé l'article, participé au choix du journal de publication.

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Résumé de l'article en français

La mélatonine a été proposée comme traitement possible des effets délétères de l'hypoxie/réoxygénation (H/R), tels que l'autophagie, l'inflammation et l'apoptose. Les grossesses pathologiques, telles que la prééclampsie, sont associées à une H/R placentaire et à une diminution de la synthèse de la mélatonine placentaire, ainsi qu'à une diminution des taux de mélatonine dans le placenta et dans le plasma maternel. Cependant, les effets de la mélatonine exogène sur l'inflammation et l'autophagie induites par les complications de la grossesse associées à l'H/R n'ont été jamais étudiés. Cette étude visait à déterminer si la mélatonine protégeait les trophoblastes villeux primaires humains contre l'inflammation et l'apoptose induites par l'H/R. Les cytotrophoblastes villeux primaires humains ont été isolés et immunopurifiés à partir de placentas de grossesses normales à terme. Ces cellules ont ensuite été exposées ou non à la mélatonine 1 mM pendant 72 h en normoxie (8% O_2), induisant ainsi une différenciation en syncytiotrophoblaste qui ont ensuite été exposés à l'H/R (0,5% O_2 pendant 4 h) ou la normoxie. L'H/R a diminué la synthèse de mélatonine endogène (de 68%) et de l'interleukine (IL)-10 (de 72%), mais a augmenté le facteur de nécrose tumorale (TNF) (de 114%), l'IL-6 (de 55%), et nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB (de 399%)), par rapport à la normoxie. Le traitement à la mélatonine a renversé les effets de l'H/R, rétablissant les taux d'IL-10, de TNF, d'IL-6 et de NFκB à ceux de normoxie. La mélatonine, ainsi que l'inhibition pharmacologique avec le pyrrolidine dithiocarbamat (PDTC) de NFκB, ont augmenté l'activation de l'autophagie, et par conséquent la survie du syncytiotrophoblaste en conditions d'H/R. Cette étude suggère que l'H/R, qui est présente dans les complications de la grossesse tel que la prééclampsie, inhibe la production de mélatonine endogène, contribuant ainsi à réduire la viabilité du syncytiotrophoblaste. Ces résultats indiquent que le traitement avec de la mélatonine exogène peut offrir une protection contre les effets délétères induits par l'H/R, améliorant ainsi la survie des cellules placentaires et par conséquent le bon développement du fœtus.

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MELATONIN MODULATES AUTOPHAGY AND INFLAMMATION PROTECTING HUMAN PLACENTAL TROPHOBLAST FROM HYPOXIA/REOXYGENATION

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SHORT TITLE: **Melatonin regulates inflammation and autophagy**

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KEYWORDS: Interleukin (IL)–6; IL-10; TNF; NFκB; syncytiotrophoblast; villous cytotrophoblasts; pregnancy; arylalkylamine N-acetyltransferase (AANAT)

HIGHLIGHTS

- Hypoxia/reoxygenation (H/R) induces pro-inflammatory responses, which inhibits melatonin synthesis in syncytiotrophoblast.
- H/R upregulates autophagy and induces apoptosis in syncytiotrophoblast.
- Under H/R, melatonin exposure inhibits the pro-inflammatory response inducing autophagy, leading to syncytiotrophoblast survival.
- Inhibition of NFκB in H/R condition upregulates autophagy, which leads to syncytiotrophoblast survival.

ABSTRACT

Melatonin has been proposed as a possible treatment for the deleterious effects of hypoxia/reoxygenation (H/R), such as autophagy, inflammation, and apoptosis. Pathological pregnancies, such as preeclampsia, are associated with placental H/R, and decreased placental melatonin synthesis as well as lower melatonin levels in the placenta and maternal plasma. However, the effects of exogenous melatonin on inflammation and autophagy induced by pregnancy complications associated with H/R await investigation. This study aimed to determine as to whether melatonin protects human primary villous trophoblasts against H/R induced autophagy, inflammation and apoptosis. Human primary villous cytotrophoblasts were isolated and immunopurified from normal term placentas. These cells were then exposed or not to 1 mM melatonin for 72 h in normoxia (8% O_2), thereby inducing differentiation into syncytiotrophoblast that were then exposed to H/R (0.5% O_2, for 4 h) or

normoxia. H/R decreased endogenous melatonin synthesis (by 68%) and interleukin (IL)-10 levels (by 72%), coupled to increased tumor necrosis factor (TNF) (by 114%), IL-6 (by 55%), and NFκB (by 399%), compared to normoxia. Melatonin treatment reversed the H/R effect, restoring IL–10, TNF and IL-6 levels to those of the normoxia condition. Melatonin, as well as NFκB inhibition, enhanced autophagy activation, consequently increasing syncytiotrophoblast survival in H/R conditions. This study suggests that H/R, which is present in pregnancy complications, inhibits endogenous melatonin production, thereby contributing to reduced syncytiotrophoblast viability. Results indicate that exogenous melatonin treatment may afford protection against H/R induced damage, thereby enhancing placental cell survival, and contributing to improved fetal outcomes.

1. INTRODUCTION

During pregnancy, physiological inflammation is necessary for optimal feto-placental unit development. However, placental inflammation is increased in abnormal pregnancies, contributing to reduced viability of placental trophoblasts, and leading to maternal-obstetrical pathologies, such as preeclampsia, preterm birth, intrauterine growth restriction (IUGR), and even fetal death [1, 2]. The mechanisms modulating trophoblastic cell survival in such disorders require clarification, with changes in oxidative stress, apoptosis, inflammation and autophagy all implicated [3-6].

Previous work indicates the utility of nonsteroidal anti-inflammatory drugs and antioxidants as treatments in pregnancy disorders, such as preeclampsia, preterm birth and IUGR. Although showing some utility, experimental studies and clinical trials show mixed results and unexpected poor outcomes, including elevated gestational hypertension and low birthweight following treatment with high doses of the antioxidants, vitamin C and E [7-11]. A recent clinical trial indicates the efficacy of a night-time administered low dose aspirin in the prophylaxis of early preeclampsia [12]. Melatonin has also been proposed as a therapeutic

option for preeclampsia and IUGR, given melatonin's utility in improving the viability of human trophoblasts exposed *in vitro* to hypoxia/reoxygenation (H/R) [13], as well as improving the mitochondrial respiration status of syncytiotrophoblast in obese women [14].

Placental villous cytotrophoblasts and syncytiotrophoblast produce melatonin and express its receptors across the duration of pregnancy [15, 16]. Over the course pregnancy, maternal plasma levels of melatonin gradually increase reaching their peak near parturition. Pregnant women with severe preeclampsia have lower plasma and placental levels of melatonin [17-19]. Melatonin has recently been shown to have efficacy in the management of preeclampsia, with melatonin decreasing levels of prematurity and lowering the need for antihypertensive treatment [20]. However, the mechanism involved in the therapeutic efficacy of treatments like melatonin in preeclampsia still await clarification. Given that optimized placenta development is crucial to maternal and fetal well-being, the role of melatonin in the placenta is important to determine.

Inflammation is one of the early pathogenic events occurring during the progression of preeclampsia. One of the defense mechanisms against excessive inflammation is autophagy activation, which is an important contributor to the restoration of cell homoeostasis. Melatonin has been shown to regulate autophagy in different cell types, and it awaits investigation as to whether melatonin regulates autophagy in placental cells [21]. It is also unknown as to whether inflammation modulates placental autophagy and endogenous melatonin synthesis. This study therefore aimed to investigate the effect of H/R on the synthesis of endogenous melatonin and the concomitant effect of H/R and exogenous melatonin on the levels of inflammation and autophagy cell survival on human primary villous trophoblastic cells. It was also investigated as to how the inhibition of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a major inflammation modulator under H/R conditions, modulates autophagy and trophoblast cell death.

2. MATERIAL AND METHODS

2.1. Isolation and culture of human primary villous trophoblasts

This study was approved by the ethical committee of CHUM-St-Luc Hospital (Montreal, QC, Canada). Term placentas were obtained immediately after spontaneous vaginal deliveries from uncomplicated pregnancies with informed patient consent. Villous cytotrophoblasts were isolated, based on the classic trypsin-DNase/Percoll method [22] and modified in our laboratory [16, 23]. Briefly, the placenta was manually minced, weighed, and exposed to four consecutive digestion baths containing trypsin and DNAse. The supernatant of each bath was collected, centrifuged and pellets were pooled. The pool of pellets was added to a Percoll column and centrifuged. The layer containing 40 - 50% of the density, which contained mainly trophoblasts, was collected [22, 23]. Following the isolation, mononuclear villous trophoblasts were purified by immunomagnetic labeling using autoMACS™ (Myltenyi Biotec, Santa Barbara, CA), as described previously. All cell preparations used in this study had a purity of at least 98% of villous trophoblasts after cell sorting [24].

Villous cytotrophoblasts were seeded in 6-well plates at a density of 3.0×10^6 cells/ml and promptly exposed to normoxia [25] (8% O_2, 5% CO_2, 87% N_2) in Modular Incubator Chambers (Billups-Rothenberg, San Diego, CA) for 6h to ensure cell adherence. Cells were washed thoroughly with warm media containing 10% FBS and then maintained under normoxia for 72 h to allow the differentiation of mononuclear villous cytotrophoblasts into syncytiotrophoblast. Every 24 h, the medium was changed and trophoblasts were either treated with melatonin 1 mM (Sigma-Aldrich, Oakville, Canada) or vehicle control (dimethyl sulfoxide (DMSO) 0.1%). After 72 h of culture under normoxia, cells were either exposed to normoxia for another 22 h or exposed to hypoxic conditions (0.5% O_2, 5% CO_2, 94.5% N_2) for 4h hours followed by 18 h of normoxia, creating a hypoxia/reoxygenation effect with or without melatonin treatment (**Fig. 1**) [13]. Normoxia mimics the concentration of oxygen in the

intervillous space of the placenta during the second and the third trimesters of pregnancy. H/R mimics *in vitro* the variation of oxygen in the intervillous space of placentas from patients with pregnancy disorders, such as preeclampsia [26, 27]. If not treated with melatonin, trophoblasts were treated at 72 h of culture with pyrrolidine dithiocarbamate (PDTC) 25 µM (Tocris, Bristol, UK) [28, 29], rapamycin 300 nM (Tocris) [30] or lipopolysaccharide (LPS; Sigma-Aldrich) 1 µg/mL [31], which are, respectively, an NFκB inhibitor, a positive control for autophagy, and a positive control for inflammation.

2.2. Human chorionic gonadotropin (hCG) secretion

To determine the functional differentiation of the trophoblast cells exposed to DMSO vehicle or melatonin 1mM, in normoxia or H/R conditions, the secretion of human chorionic gonadotropin (hCG) was analyzed. Briefly, cell culture media were collected and centrifuged every 24 h, and then were stored at −20°C until assayed. The secretion of hCG was evaluated by enzyme-linked immunosorbent (ELISA) assay according to manufacturer's instructions (IBL International, Toronto, ON, Canada). hCG medium content was measured at 450 nm using the Spectra Max M5 (Molecular Devices).

2.3. Immunofluorescence

Human villous trophoblasts were fixed with methanol at – 20°C for 20 min and then permeabilized with 0.1% Triton X-100 for 5 minutes. Samples were incubated with 2% FBS in PBS for 1h to eliminate nonspecific antigen binding epitopes. Cells were stained overnight (at 4°C) with a rabbit microtubule-associated protein 1A/1B light chain 3B (LC3-B) antibody (Sigma-Aldrich; 1:150, overnight, 4°C) or a mouse desmoplakin (Abcam, Cambridge, MA) (1:300). Secondary antibodies (1:500) were coupled to IgG Fab2 Alexa Fluor 488 (anti-mouse) and IgG Fab2 Alexa Fluor 594 (anti-rabbit). Cell nuclei were stained with 4',6-

diamidino-2-phenylindole (DAPI) present in the antifade reagent ProLong™ Gold (Thermo Fisher Scientific). Immunofluorescence images were obtained with a Nikon A1R+ confocal microscopic laser equipped with a spectral detector and analyzed using NIS-elements software (version 4).

2.4. Immunoblotting

Human villous trophoblasts were rinsed once with PBS and lysed with ice-cold modified radioimmunoprecipitation (RIPA) buffer (50 mmol/l Tris-HCl pH 7.4, 1% NP-40, 0,25% Na-deoxycholate, 150 mmol/l NaCl and 1 mmol/l EDTA) containing protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were extracted on ice with continuous mixing for 30 min and then sonicated. The homogenate was then centrifuged at 14,000 g for 10 mins at 4°C. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Waltham, MA). For analysis, samples (10 or 12 µg protein) were separated on Mini-PROTEAN® TGX™ Precast Protein Gels (4–15% precast polyacrylamide gel) (Bio-Rad, Saint Laurent, Canada), followed by transfer to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) and immunoblotting with the indicated antibodies: phospho (Thr 31) aralkylamine N-acetyltransferase (AANAT) (Millipore, Etobicoke, Canada; 1:2000 overnight, room temperature); hypoxia-inducible factor 1-alpha (HIF-1α; R&D systems, Minneapolis, MN; 1:1000 overnight, 4°C); Phospho (Ser 276) nuclear factor kappa-light-chain-enhancer of activated B cells (NFκBp65; Millipore; 1:1000 overnight, 4 °C); mouse NFκB p65 (R&D systems; 1:400 overnight, 4°C); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα; Cell Signaling, Danvers, MA; 1:400 overnight, 4°C); Beclin-1 (Cell Signaling; 1:1000 overnight, 4°C); rabbit LC3-B (1:2000; Sigma-Aldrich, 1 h, 4°C); Poly ADP-Ribose Polymerase (PARP-1; Cell Signaling; 1:1000 overnight, 4°C); Cleaved PARP (Cell Signaling; 1:1000 overnight, 4°C); Caspase 3 (Cell Signaling; 1:300 overnight, 4°C); Cleaved-caspase 3 (Cell Signaling; 1:500

overnight, 4°C). Blots were developed with enhanced chemiluminescence reagent (Bio-Rad). Protein levels were expressed as a ratio of a specific band density and total protein stained using Pierce™ Reversible Protein Stain Kit (Pierce Biotechnology) as previously described [32, 33]. Bands were quantified using Image Lab software 5.1 (Bio-Rad).

2.5. Melatonin assay

Melatonin release in the supernatant obtained at 96 h of villous trophoblast culture was measured with a melatonin ELISA kit (IBL #RE54021) from Hamburg, Germany. The regular method provided by the kit, activating columns with HPLC-grade methanol with centrifugation (1 min at 120 x g) followed by evaporation of the sample with a SpeedVac concentrator. Detection limit for melatonin in this assay is 1.6 pg/mL. Cross-reactivity of other substances < 2.2%. Melatonin content was calculated against standard values provided by the manufacturer using the 4-parameter logistic curve and normalized to protein content of the supernatant.

2.6. Analysis of Cytokines in Cell Culture Supernatants.

Villous trophoblast cell culture supernatants were filtered with Amicon centrifugal filter devices (Millipore). The chosen concentration factor was 8–fold using a filter suitable to retain any molecule greater than 3 kDa. Then, the concentration of tumor necrosis factor (TNF), interleukin (IL)–6, and IL–10 levels were analyzed using multiplex ELISA (Milliplex Map, Millipore). The chosen panel (HCYTOMAG-60K) contained antibodies linked to magnetic beads and the analyses were determined using the Luminex xMAP technology (Luminex, Austin, TX).

2.7. siRNA transfection

For knockdown of endogenous AANAT, human AANAT-specific short interfering RNA (siRNA) or scrambled RNA as control was mixed with Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA) in serum-free Opti-MEM medium for 15 min. The mixture was loaded onto villous trophoblasts in culture after 24 h and 48 h of culture in normoxia. Opti-MEM medium was renewed every 24 h. The human AANAT-specific siRNA and scrambled RNA were purchased from Qiagen (Germantown, MD).

2.8. Lactate Dehydrogenase (LDH) Assay

LDH release into villous trophoblast cell culture supernatants was detected by colorimetric ELISA, using the cytotoxicity detection kit according to manufacturers' instructions (Roche, Laval, QC, Canada).

2.9. Statistical analysis.

All data represent at least three different primary villous trophoblast cell cultures from different placentas. Statistically significant differences ($p \leq 0.05$) of parametric results were determined by one-way analysis of variance (ANOVA) and identified by the *post hoc* test of Student-Newman-Keuls. Non-parametric data were analyzed using Kruskal-Wallis test, followed by *post hoc* Dunn's test. Data were analyzed using GraphPad Prism (version 5.04).

3. RESULTS

3.1. H/R decreases endogenous melatonin synthesis in syncytiotrophoblast.

Human primary villous cytotrophoblastic cells were kept in normoxia for 72 h, to allow their differentiation into syncytiotrophoblast. The increased hCG release (by 1.7–fold) after 72 h of culture confirms the functional differentiation (**Fig. 1b**). The exposure to H/R led to a 1.3–fold decrease in hCG levels, compared to cells exposed to normoxia (**Fig. 1b**). Cells treated with melatonin during the 96 h of culture and exposed to H/R showed similar hCG levels as cells kept in normoxia. The morphological differentiation (fusion) of mononuclear villous cytotrophoblasts into the multinucleated syncytiotrophoblast was also achieved, as demonstrated in the **Figure 1c**. Given that placental melatonin is decreased in pregnancies with complications, we tested as to whether syncytiotrophoblast cells in H/R show altered melatonin synthesis. As shown in the **Figures 2a, b,** human syncytiotrophoblast is able to synthetize and release melatonin in the culture medium. The exposure to H/R decreased melatonin release by 2.4–fold. To confirm that endogenous melatonin synthesis is impaired by H/R, the activation of the enzyme AANAT, the main mediator melatonin synthesis, was measured, as indicated by Thr 31 phosphorylation [34]. Results show H/R to decrease the levels of phosphorylated AANAT (by 3.1–fold) (**Fig. 2b, c**). The knockdown of AANAT by siRNA resulted in 2.5–fold reduction of melatonin levels in the syncytiotrophoblast culture media (**Fig. 2d, e**). Treatment of the cells with 1 mM melatonin restored the levels of melatonin in the supernatant, but had no effect on the levels of phosphorylated AANAT (**Fig. S1**). LPS was used as a positive control of inflammation. LPS, which is represented by the dashed line, decreased melatonin (**Fig. 2a**) and phospho–AANAT protein levels (**Fig. 2b, c**). The similar pattern of phospho–AANAT in H/R and under LPS induction may indicate the role of inflammation in H/R effects.

3.2. H/R induced hypoxia in syncytiotrophoblast indicated by increased HIF-1α protein levels

To further determine the effect of H/R *in vitro* on primary syncytiotrophoblast, the protein levels of the transcription factor HIF-1α were measured. In normoxia, HIF-1α is ubiquitinated and degraded by the proteasome. Whereas, in H/R conditions the transcription factor is stable and can upregulate genes whose promoters contain hypoxia response consensus sequences, thereby inducing angiogenesis, vasodilatation and apoptosis [35]. In H/R, HIF-1α is increased 4.0-fold in syncytiotrophoblast, compared with normoxia. Melatonin was able to restore the levels of HIF-1α in H/R to levels found normoxia (**Fig. 3**).

3.3. Melatonin blocks the phosphorylation of NFκB Ser 276 induced by H/R

The activation of NFκB in syncytiotrophoblasts was also investigated. Following H/R, NFκB protein level increased **(Fig. 4a**). In homoeostasis, NFκB is retained in the cytoplasm by IκB proteins. Under stress, IκB proteins, such as IκBα, are phosphorylated and promptly degraded by the proteasome [36]. In syncytiotrophoblast exposed to H/R, IκBα was dramatically reduced (**Fig. 4a**). Following the degradation of IκB, NFκB is phosphorylated on Ser 276 leading to its interaction with the transcriptional coactivator CBP/p300. H/R increased Ser 276 phosphorylation (by 4.4–fold), with this phosphorylation being inhibited by melatonin treatment (**Fig. 4a, b**). These results suggest that NFκB is activated in H/R and that melatonin can inhibit the activation of this transcription factor.

3.4. Melatonin reverses the increased TNF and IL-6 and the decreased IL-10 levels induced by H/R in syncytiotrophoblast

To investigate if H/R induces inflammation in syncytiotrophoblast, TNF, IL–6 and IL–10 levels were measured. H/R, versus normoxia, increased syncytiotrophoblast TNF release

by 2.1-fold (**Fig. 5a**). Syncytiotrophoblast transfected with siAANAT showed similar TNF levels as cells transfected with scrambled sequences (**Fig. 5d**). In contrast, H/R-exposed cells treated with melatonin showed TNF levels equivalent to those observed in normoxia. Similarly, IL–6 levels, important drivers of inflammation in preeclampsia [37], were increased by 1.5-fold under H/R conditions (**Fig. 5b**). Transfection of siAANAT enhanced IL-6 increase by 2.6-fold in normoxia and 1.7-fold in H/R (**Fig. 5e**). Melatonin treatment significantly decreased IL–6 levels by 1.8-fold under H/R, thereby restoring IL-6 levels to those of normoxia (**Fig. 5b**). In contrast to its effects on pro-inflammatory cytokines, H/R, versus normoxia, decreased the release of the anti-inflammatory cytokine, IL–10, in syncytiotrophoblast by 3.5-fold (**Fig. 5c**). This is supported by the low levels of IL-10 in preeclamptic patients [38]. Melatonin synthesis inhibition with siAANAT and its control (scrambled) produced similar reductions in IL-10 levels in H/R conditions, compared to cells exposed to normoxia (**Fig. 5f**). Melatonin treatment, on the other hand, increased IL–10 levels by 2.6–fold in normoxia and reversed H/R effects on this cytokine. TNF and IL–10 levels in H/R-exposed trophoblasts is comparable to the levels found with LPS (a positive control of inflammation), perhaps indicative of the inflammation occurring in syncytiotrophoblast being linked to intermittent oxygen concentrations. To determine if the effect of melatonin on TNF and IL-10 release is receptor-mediated, syncytiotrophoblasts were treated with luzindole (1mM) [39] or luzindole and melatonin in both normoxia and H/R conditions. Luzindole is a strong antagonist of the melatonin G protein-coupled membrane receptors (MT1 and MT2)[40]. The levels of both TNF and IL-10 of syncytiotrophoblast treated with luzindole or luzindole and melatonin were similar (data not shown) to cells treated with melatonin alone, namely decreased TNF and a slight increase of IL-10 under H/R conditions. This could indicate that the effects of melatonin on TNF and IL-10 are not dependent of its membrane receptors (MT1 and MT2). However, it should be noted that luzindole is also an antioxidant, and so may enhance the receptor-independent beneficial effects of melatonin [39, 41].

3.5. H/R induces autophagy in syncytiotrophoblast

Previous studies have shown that hypoxic conditions led to activation of autophagy in human primary villous cytotrophoblasts[30, 42]. Beclin-1 is a major activator of macroautophagy, referred herein simply as autophagy [43]. H/R, with and without melatonin, significantly upregulated Beclin-1 (**Fig. 6a, b**). LC3B has an important role in selecting cargos (autophagy carriers containing protein aggregates and organelles) for degradation [4]. Once activated, LC3B is lipidated into its active form: LC3B-II, which has a lower molecular weight (16 kDa) and can be visualized as cytosolic LC3B puncta [5]. The active form (LC3B-II) is also significantly increased in syncytiotrophoblast exposed to H/R, with or without melatonin treatment, compared to normoxia (**Fig. 6a, c, d**). The amount of activation of LC3B in H/R conditions is comparable to the activation obtained with the classic autophagy inducer rapamycin (by 2.6–fold; **Fig. 6c**) [3].

3.6. Inhibition of NFκB has no effect on melatonin synthesis and increases autophagy and TNF in syncytiotrophoblast under H/R

To elucidate the role of NFκB on melatonin synthesis and autophagy in syncytiotrophoblast, we inhibited the activation of this transcription factor with its specific inhibitor, PDTC (25 μM) [28, 29] (**Fig. 7**). As demonstrated in Fig. 7a, PDTC dramatically inhibited the phosphorylation of NFκB under H/R conditions. NFκB inhibition significantly increased the phosphorylation level of AANAT by 1.75–fold, compared to DMSO vehicle control, in normoxia. However, NFκB inhibition showed no effect in H/R (**Fig. 7a, b**). Melatonin level was not modulated by PDTC treatment (data not shown).

The PDTC induction in normoxia had no effects on trophoblastic expression of Beclin-1 and LC3B-II. In H/R, PDTC significantly increased the expression of Beclin-1 and LC3B-II, compared to normoxia and H/R without PDTC (**Fig. 7c, d**). Similarly, in normoxia, TNF levels were not modulated by PDTC treatment, however, in H/R, PDTC increased

significantly TNF levels (by 3.2–fold) (**Fig. 8c**). Neither syncytiotrophoblast exposed to H/R nor those exposed to normoxia had their levels of IL-10 modulated by PDTC (data not shown).

3.7. Inhibition of NFκB mimics the protective effect of melatonin against H/R-induced inflammation and apoptosis in syncytiotrophoblast.

As previously described, melatonin inhibits trophoblast apoptosis under H/R conditions[13]. Melatonin treatment, and the inhibition of NFκB, in H/R reduced the cleavage of the apoptosis effectors, Caspase 3 and PARP, compared to syncytiotrophoblast exposed to H/R (**Fig. 8a, b**). Also, trophoblasts exposed to LPS had higher levels of cleaved–PARP compared to all other conditions (by 3.05–fold), indicating that inflammation can play a role in the activation of cell death in villous trophoblasts. Finally, H/R is able to increase the release of the cytoplasmic LDH in the culture media, a known marker of cell membrane disruption in cases of cell death. However, cells exposed to H/R and treated with melatonin or PDTC showed LDH release levels comparable to those found in normoxia (**Fig. 8d**) [44].

4. DISCUSSION

The activation and regulation of inflammatory pathways is necessary in the control of villous trophoblast survival and differentiation. However, under abnormal oxygenation during the pregnancy, excessive inflammation is damaging [45]. This study shows melatonin to significantly regulate inflammation and autophagy in H/R conditions, thereby reducing levels of apoptosis. Our *in vitro* H/R model has previously been shown to mimic the molecular modifications observed in placentas from pathological pregnancy, such as preeclampsia, as indicated by the levels of intrinsic apoptosis and oxidative stress in primary syncytiotrophoblast [13]. In the present study, this *in vitro* H/R model was shown also to

parallel the inflammatory status and autophagy that are readily detectable in placental and maternal samples from patients with complications of pregnancy [46].

Despite the increase of the active form of the autophagy marker, LC3B-II, in syncytiotrophoblast exposed to H/R, autophagy activation was insufficient to avoid the raised levels of cell death. As recently reviewed by Nakashima et al., [47] the challenging conditions faced by trophoblasts during placentation may necessitate the activation of autophagy during the establishment of adequate levels of oxygen in the placental intervillous space. In fact, lower levels of autophagy during the first trimester of pregnancy are associated with shallow trophoblastic invasion, a major factor in the development of preeclampsia [2, 48]. Towards the end of pregnancy, preeclampsia is associated with high levels of placental autophagy, including in the syncytiotrophoblast [49, 50]. The current results corroborate previous studies showing the activation of autophagy in *in vitro* models of human villous trophoblasts exposed to lower oxygen levels [30, 42]. As expected, the activation of the autophagic pathway was not modulated by melatonin in normoxia [25]. In contrast, in H/R condition melatonin generated higher levels of Beclin–1 and activation of LC3B, leading, consequently, to lowered levels of cell death. Such data indicate that, under H/R conditions, autophagy, by itself, is unable to rescue the cell viability. However, the protection provided by melatonin can improve the effects of autophagy to enhance cell survival rates. In support of this, treating syncytiotrophoblast with PDTC, an NFκB inhibitor [51], significantly increases autophagy in H/R and abolish aberrant levels of cell death [28].

In preeclampsia, both plasma and placental levels of TNF and IL–6 are dramatically increased, whilst levels of the anti-inflammatory cytokine, IL–10, are decreased [1, 52]. Such an increase in the ratio of pro-/anti-inflammatory cytokines can lead to macrophage activation and vasoconstriction, a hallmark of preeclampsia severity [38, 53, 54]. The H/R induction of TNF and IL-6 and lowering of IL-10 in syncytiotrophoblast model used in the current study indicates its suitability as an *in vitro* model of preeclampsia. Melatonin reverses the increase in TNF and IL-6 and the decreased IL-10 induced by H/R, whilst siAANAT increases the

levels of the pro-inflammatory cytokine, IL-6, in both normoxia and H/R conditions. The inhibition of melatonin synthesis by AANAT knockdown resulted in a significant decrease in melatonin levels in normoxia, but not in H/R. The downregulation of AANAT by H/R could explain the reason why a significant reduction of melatonin was not observed in villous trophoblast cells exposed to siAANAT. This suggests that, during pregnancy, endogenous melatonin has an inhibitory effect on the release of IL-6, a cytokine that promotes endothelial cell dysfunction in the preeclamptic placenta [54]. Hence, the inhibitory effects of melatonin on pro-inflammatory cytokines may also be related to the modulation of placental blood flow. As reviewed by Marseglia *et al.* (2016) melatonin could protect both pregnant women and neonates in cases of pregnancy disorders [55].

The results of the current study indicate that melatonin's suppression of TNF and IL-6 and induction of IL-10 is mediated via NFκB inhibition. Melatonin had no effect on the cytosolic levels of NFκB and IκBα, but reduced the phosphorylation of NFκB. NFκB can also have differential effects on the AANAT and melatonin synthesis in different cell types, which is dependent on the different NFκB subunits in these cells[56, 57]. To clarify as to whether NFκB activation is implicated in the down-regulation of phosphorylated AANAT in our model, syncytiotrophoblast were exposed to PDTC that prevents IκBα ubiquitylation, leaving NFκB in a non-active state [29]. In normoxia, PDTC increases the levels of melatonin and phospho-AANAT, indicating that in trophoblasts, as in pinealocytes, NFκB activation inhibits melatonin synthesis[58]. In H/R, though, the inhibition of NFκB did not rescue endogenous melatonin synthesis. However, similar to melatonin, the inhibition of NFκB in H/R dramatically increased levels of autophagy in association with lower cell death. It is therefore plausible that hypoxic conditions generate excessive NFκB activation, consequently leading to inflammation and eventual trophoblast death. Clockie, *et al*, have shown that pineal AANAT is regulated by miRNAs, which impair its stability[59]. Likewise, H/R conditions also upregulate the expression of miR-325-3p, which suppresses AANAT in the pineal gland of rats[60, 61]. The expression of the miR-325-3p is increased in endothelial cells exposed to low doses of TNF

for 96 h [62]. In our study, when syncytiotrophoblast cells were treated with PDTC and challenged with H/R, TNF levels are increased coinciding with lower levels of phosphorylation of AANAT, in comparison to syncytiotrophoblast cells treated with PDTC in normoxia. The mechanism involved in the modulation of TNF and AANAT in villous trophoblast exposed to H/R and PDTC requires further investigation

H/R increased levels of HIF-1α, with melatonin treatment restoring HIF-1α protein levels to normoxia level. This is in accord with a recent study that suggests melatonin inhibits HIF-1α protein and mRNA in cardiac progenitor cells [63]. This modulation of oxygenation by H/R impairs melatonin production in human primary syncytiotrophoblast and significantly increases inflammation, autophagy and cell death. Further, we provide a unique insight into melatonin as a cytoprotective molecule, being able to protect against increased levels of inflammation and apoptosis as induced by H/R in primary human villous trophoblasts. Our study suggests that H/R, which is observed in pregnancy complications, inhibits the placenta's endogenous melatonin production, resulting in lower melatonin levels in the maternal blood and placenta, as observed in preeclamptic women. In conclusion, the data indicate that exogenous melatonin treatment could afford protection against H/R induced damage, thereby enhancing placental cell survival, and consequently improving pregnancy and fetal outcomes (**Fig. 9**).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

All co-authors contributed to the design of the study protocol. LSF was involved in data acquisition for the section results. LSF, was actively involved in the writing of the manuscript and CV assisted in drafting the manuscript. Finally, all co-authors provided critical revisions and provided their final approval for the manuscript's publication in Journal of Pineal Research. This manuscript is not under review elsewhere and all authors are in agreement with the contents of this manuscript.

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Figure Legends

Fig. 1: Experimental design and differentiation of primary villous cytotrophoblasts into syncytiotrophoblast. (a) Primary villous cytotrophoblasts cells were cultured under normoxia (8% O_2; 5% CO_2; 87% N_2) for 72 h to allow their differentiation into syncytiotrophoblast. The cells were then exposed to normoxia or hypoxia (4 h)/reoxygenation (18 h) (0.5% O_2; 5% CO_2; 94.5% N_2). Cells were treated or not (DMSO vehicle control, 0.1%) every 24 h with 1 mM melatonin. Lipopolysaccharide (LPS) (1 µg/mL) was used as a positive control for inflammation; pyrrolidine dithiocarbamate (PDTC) (25 µM) as an inhibitor of NFκB activation; rapamycin (RAP) (300 nM) as a positive control of autophagy. (b) The release of hCG was measured using an ELISA kit. Data shown are average ± SD. (**$P \leq 0.01$), n = 4 different placentas. (c) Representative immunofluorescence images; syncytiotrophoblast cultured under normoxia or H/R treated with 1mM melatonin or DMSO 0.1%, as control, were fixed in methanol, stained with desmoplakin for membranes (green), counterstained with 4',6-diamidino-2-phenylindole (DAPI) (nucleus staining blue), and observed by confocal microscopy at 20x magnification. N; normoxia, H/R; hypoxia/reoxygenation. Scale bar represents 10 µm.

Fig. 2: Hypoxia/reoxygenation inhibits villous trophoblast synthesis of melatonin. (a) Melatonin content (pg/ mg of protein) analyzed by ELISA. Data shown are average ± SD, n = 3-4 different placentas. A statistically significant difference between normoxia and hypoxia/reoxygenation (H/R) (Student's t-test, $*P \leq 0.05$; $**P \leq 0.01$). (b) Phospho-AANAT protein (P-AANAT)/total protein levels in villous trophoblasts under normoxia or hypoxia/reoxygenation. Dashed lines indicate the average of melatonin content and P-AANAT under lipopolysaccharide (LPS) in normoxia. LPS at a concentration of 1 µg/mL was added for the final 24 h of the culture period as a positive control of inflammation. Data shown are average ± SD of four independent experiments. A statistically significant difference between normoxia and hypoxia/reoxygenation (H/R) (Student's t-test, $*P \leq 0.05$). (c - e) Representative western blot of phosphorylated (Thr 31) aralkylamine N-acetyltransferase (P-AANAT), the key- enzyme of melatonin synthesis. Total protein amounts were measured by staining with MemCode reversible protein stain. The relative molecular mass (kDa) is indicated to the right of the blot. (d) Villous trophoblasts were transfected with siRNA of AANAT (siAANAT) or scrambled RNA as a control, which was transfected at 24 h and also at 48 h of culture.

Fig. 3: Melatonin protects villous trophoblast against hypoxia/reoxygenation-induced increased HIF-1α protein level. Primary villous cytotrophoblasts were treated for 96 h with 1 mM melatonin or dimethyl sulfoxide ((DMSO) 0.1%: vehicle control) under normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia/reoxygenation (0.5% O_2; 5% CO_2; 94.5% N_2) conditions. (a) Hypoxia-inducible factor 1-alpha (HIF-1α) protein level was detected by immunoblotting using a polyclonal antibody. The relative molecular mass (kDa) is indicated to the right of the blot. Mem code stain of total protein was used to normalize protein expression. (b) Bar graph represents the relative optical density of HIF-1α normalized with total protein. Data shown are average ± SD of five different placentas and were analyzed using ANOVA, followed by Student-Newman-Keuls ($* P \leq 0.05$). The dashed line indicates the average of HIF-1α

protein level in villous trophoblasts treated with 1 µg/mL lipopolysaccharide (LPS) under normoxia conditions, as a positive control of inflammation.

Fig. 4: Melatonin prevents hypoxia/reoxygenation-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB p65) SER-276 phosphorylation in villous trophoblast. Primary villous cytotrophoblasts were treated for 96 h with 1 mM melatonin or dimethyl sulfoxide ((DMSO) 0.1%: vehicle control) under normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia/reoxygenation (0.5% O_2; 5% CO_2; 94.5% N_2) conditions. (a) Phosphorylation of the Ser 276 of NFκB p65 was detected by immunoblotting using a polyclonal phospho-specific antibody. Total protein expression of NFκB p65 and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) were detected by immunoblotting using monoclonal antibodies. The relative molecular masses (kDa) are indicated to the right of the blot. Mem code stain of total protein was used to normalize protein expression. (b) Bar graphs represent the relative optical density of p-NFκB p65 (Ser 276) normalized with that of total NFκB p65. Data shown are average ± SD of five different placentas and were analyzed using ANOVA, followed by Student-Newman-Keuls. (** $P \leq 0.01$; *** $P \leq 0.001$). The dashed line indicates the average of HIF-1α protein level in villous trophoblast treated with 1 µg/mL lipopolysaccharide (LPS) under normoxia conditions, as a positive control of inflammation.

Fig. 5: Melatonin prevents the increase of inflammatory mediators induced by hypoxia/reoxygenation in villous trophoblast. (a - f) Tumor necrosis factor (TNF), Interleukin-6 (IL–6) and IL-10 supernatant content was measured using a Multiplex ELISA detection system in villous trophoblast cell-free supernatants after 96 h of culture. (a and d) Levels of soluble TNF, * $P \leq 0.05$ (N = 3 – 6); (b and e). Levels of soluble IL–6, * $P \leq 0.05$, ** $P \leq 0.01$ (N = 4 – 6); (c and f). Levels of soluble IL–10, * $P \leq 0.05$ (DMSO normoxia,

compared to all other groups), ** $P \leq 0.01$ (N = 3 – 5). The dashed line indicates the average of the inflammatory mediators in villous trophoblast treated with 1 µg/mL lipopolysaccharide (LPS) under normoxia conditions, as a positive control of inflammation.

Fig. 6: Hypoxia/reoxygenation and melatonin activate autophagy in villous trophoblasts. (a) Primary villous cytotrophoblasts were treated for 96 h with 1 mM melatonin or dimethyl sulfoxide ((DMSO) 0.1%: vehicle control) under normoxia (8% O_2; 5% CO_2; 87% N_2) and hypoxia/reoxygenation (0.5% O_2; 5% CO_2; 94.5% N_2) conditions. Beclin-1 and LC3B protein expression were detected by immunoblotting using polyclonal antibodies. The relative molecular mass (kDa) is indicated to the right of the blot. Bar graphs represent the relative optical density of Beclin-1 (b) and LC3B-II (c) with total protein. Under normoxia conditions, lipopolysaccharide ((LPS) 1 µg/mL) or rapamycin ((RAP) 300 nM) were added for the final 24 h of the culture period as a positive control of inflammation and autophagy, respectively. The dashed lines indicate the average of Beclin-1 and LC3B-II expression under LPS induction. Data shown are average ± SD of five independent experiments and were analyzed using ANOVA, followed by *post hoc* test Student-Newman-Keuls (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). (d) Representative immunofluorescence images; syncytiotrophoblast cultured under normoxia or H/R treated with 1mM melatonin or not (dimethyl sulfoxide (DMSO) 0.1%) were fixed in methanol, stained for LC3B (red), counterstained with 4',6-diamidino-2-phenylindole (DAPI) (nucleus staining blue), and observed by confocal microscopy at 20x magnification. N; normoxia (DMSO 0.1%, melatonin 1mM, or rapamycin 300nM). H/R; hypoxia/reoxygenation (DMSO 0.1%, melatonin 1mM). Scale bar represents 10 µm.

Fig. 7: Effect of inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB p65) on melatonin synthesis and autophagy in villous trophoblast. Primary villous cytotrophoblasts were maintained during 96 h under normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia/reoxygenation (0.5% O_2; 5% CO_2; 94.5% N_2) conditions. Pyrrolidine dithiocarbamate ((PDTC) 300 nM) was added for the final 24 h of the culture period as an inhibitor of NFκB. Total protein expression of NFκB p65 and Ip65 were detected by immunoblotting using monoclonal antibodies. Phospho-NFκB (Ser 276) and Phospho-AANAT (Thr 31) were detected by immunoblotting using polyclonal phospho-specific antibodies. Beclin-1 and LC3B protein expression were detected by immunoblotting using polyclonal antibodies. The relative molecular mass (kDa) is indicated to the right of the blot. Bar graphs represent the relative optical density of Phospho-AANAT (b), Beclin-1 (c), and LC3B-II (d) with total protein. Data shown are average ± SD (N=3-4); were analyzed using ANOVA followed by *post hoc* test Student-Newman-keuls (*$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Fig. 8: Effect of inhibition of NFκB on TNF levels and apoptosis in villous trophoblast.

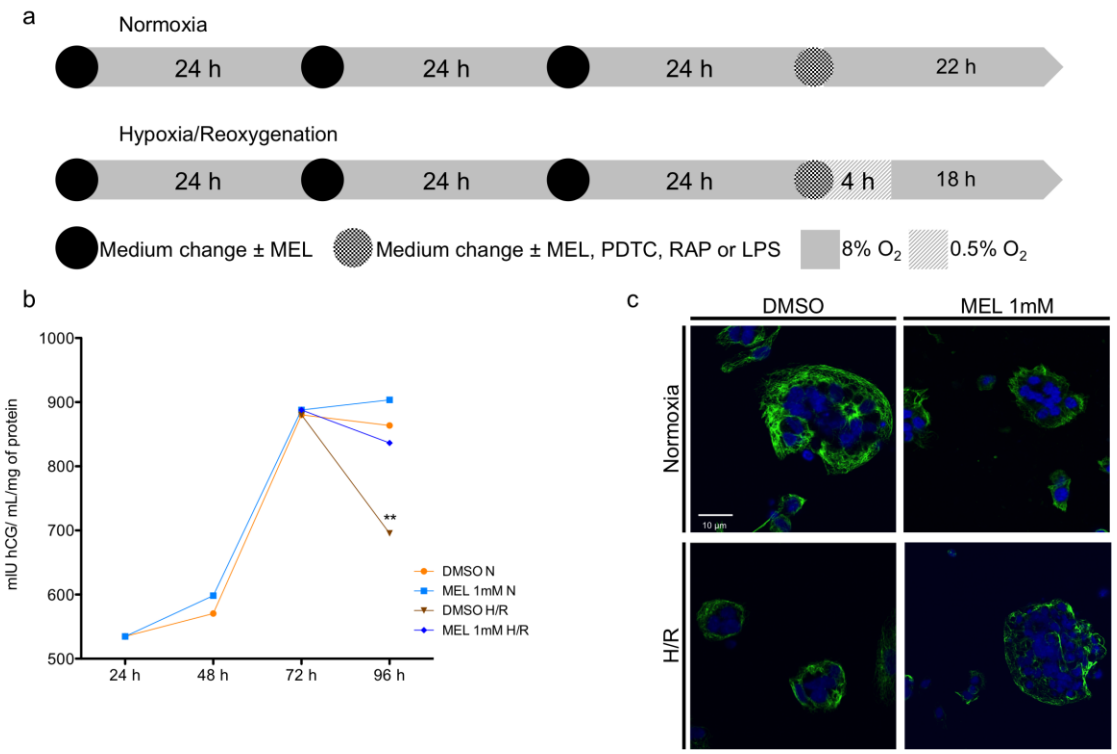
Primary villous cytotrophoblasts were maintained during 96 h under normoxia (8% O_2; 5% CO_2; 87% N_2) and hypoxia/reoxygenation (0.5% O_2; 5% CO_2; 94.5% N_2) conditions. Pyrrolidine dithiocarbamate ((PDTC) 300 nM) was added for the final 24 h of the culture period as an inhibitor of NFκB. Total protein and the cleaved portion expression of PARP-1 were detected with a polyclonal and a monoclonal antibody, respectively. Total protein and the cleaved portion expression of Caspase-3 were detected with two monoclonal antibodies. The relative molecular mass (kDa) is indicated to the right of the blot. (b) Bar graphs represent the relative optical density of Cleaved-PARP with total protein. Data shown are average ± SD (N=4) and were analyzed using ANOVA followed by *post hoc* test Student-Newman-Keuls (* $P<0.05$). The dashed line indicates the average of cleaved-PARP under

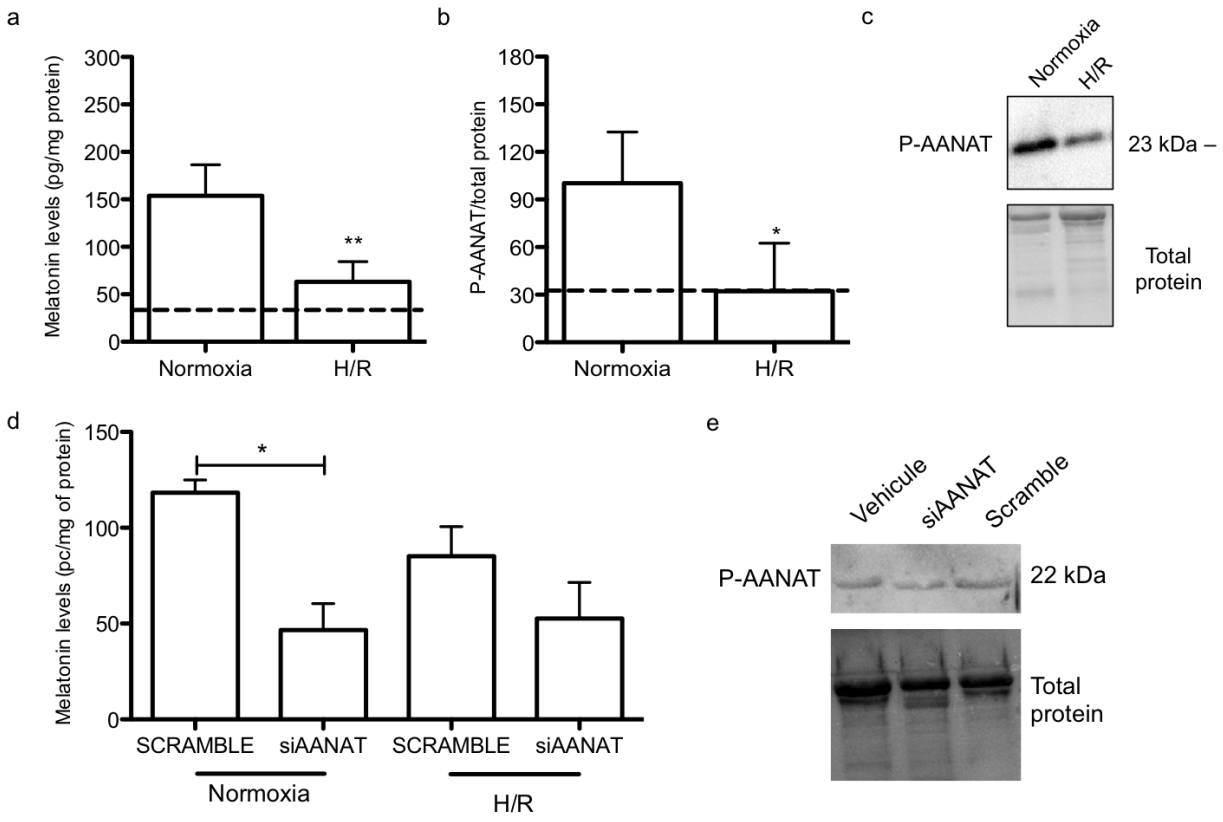
LPS induction. (c) Normalized levels of TNF detected in cell-free supernatant. Data shown are average ± SD (N=4) and were analyzed using Kruskal-Wallis followed by *post hoc* test Dunn (*$P \leq 0.05$; *** $P \leq 0.001$). (d) Leakage of cytosolic LDH into the cell culture medium. Data shown are average ± SD (N=4) and were analyzed using ANOVA followed by *post hoc* test Student-Newman-Keuls (* $P \leq 0.05$) for the measurements in the media collected at 96 h of culture.

Fig. 9: Hypothetical representation of the protective actions of melatonin against hypoxia/reoxygenation-induced villous trophoblast apoptosis. (a) In primary villous trophoblast cells, H/R increases HIF-1α protein level, autophagy and NFκB. In turn, the activation of NFκB by H/R inhibits autophagy and IL10, an anti-inflammatory cytokine that attenuates the extent of the inflammatory activation. NFκB also has a putative inhibitory effect on the activation of the enzyme AANAT, leading to a lower synthesis of melatonin. NFκB also induces HIF-1α, IL-6, TNF, and apoptosis. The increase of HIF-1α levels by both H/R and by NFκB, could lead to its translocation to the nucleus to activate genes containing a hypoxia-response element (HRE). (b) Melatonin treatment prevents the effects induced by H/R in villous trophoblast. Specifically, melatonin prevents the activation of NFκB which could be intimately related to lower HIF-1α levels as well as increased IL-10 and decreased TNF and IL-6 levels. Melatonin treatment also activates the autophagy pathway, which may contribute to decreased apoptosis. Blue and red lines indicate enhancing and inhibiting effects, respectively, of melatonin treatment.

SUPPLEMENTAL MATERIAL

Fig. S1: H/R inhibits, whereas exogenous melatonin does not affect the synthesis of melatonin. (a) Melatonin content (pg/ mg of protein) analyzed by ELISA. Data shown are average ± SD (**$P \leq 0.01$). Data were analyzed using Kruskal-Wallis, *post hoc* test Dunn. N=3-4. (b) Phospho-AANAT protein expression/total protein. Data shown are average ± SD of four independent experiments and were analyzed using ANOVA, followed by *post hoc* test Student-Newman-Keuls. (*$P \leq 0.05$). Under normoxia conditions, lipopolysaccharide (LPS) at a concentration of 1 µg/mL was added for the final 24 h of the culture period as positive control of inflammation. Dashed lines indicate the average of melatonin content and phosphorylation of AANAT under LPS induction. Representative western blot of phosphorylated (Thr 31) aralkylamine N-acetyltransferase (AANAT), the penultimate enzyme in melatonin synthesis pathway, considered the key-regulator of endogenous production. Total protein amounts were measured by staining with Mem Code reversible protein stain. The relative molecular mass (kDa) is indicated to the right of the blot.


a
Normoxia
24 h
24 h
24 h
22 h
Hypoxia/Reoxygenation
24 h
24 h
24 h
4 h
18 h
Medium change ± MEL
Medium change ± MEL, PDTC, RAP or LPS
8% O2
0.5% O2
b
mIU hCG/ mL/mg of protein
1000
900
800
700
600
500
24 h
48 h
72 h
96 h
**
DMSO N
MEL 1mM N
DMSO H/R
MEL 1mM H/R
c
DMSO
MEL 1mM
Normoxia
H/R
10 µm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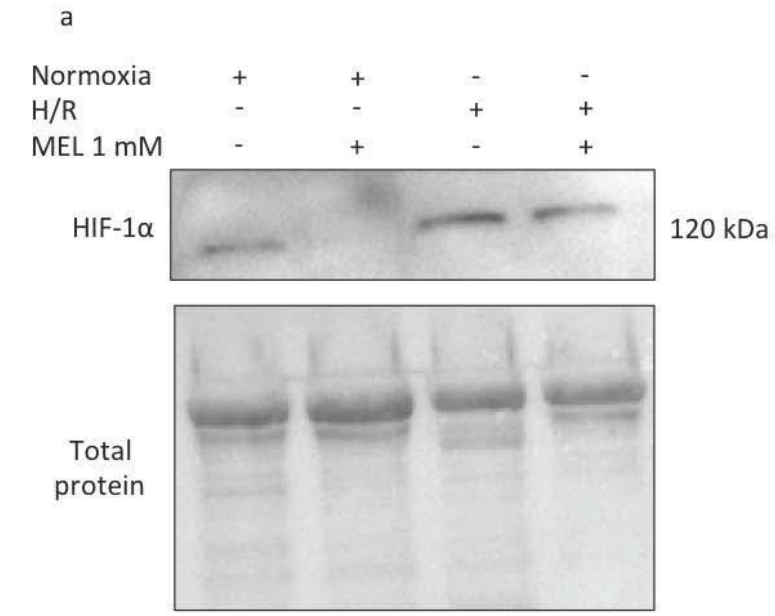
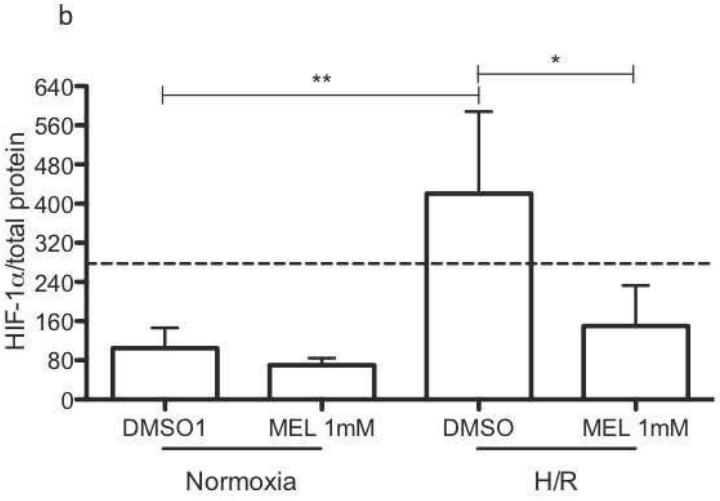
Melatonin levels (pg/mg protein)
300
250
200
150
100
50
0
**
Normoxia
H/R
b
P-AANAT/total protein
180
150
120
90
60
30
0
*
Normoxia
H/R
c
Normoxia
H/R
P-AANAT
23 kDa –
Total protein
d
Melatonin levels (pc/mg of protein)
150
100
50
0
*
SCRAMBLE
siAANAT
SCRAMBLE
siAANAT
Normoxia
H/R
e
Vehicule
siAANAT
Scramble
P-AANAT
22 kDa
Total protein





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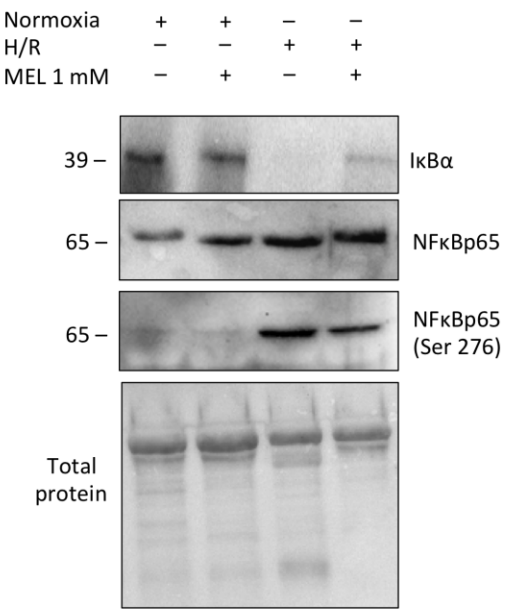
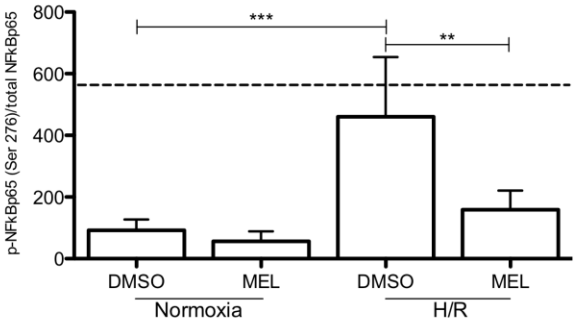
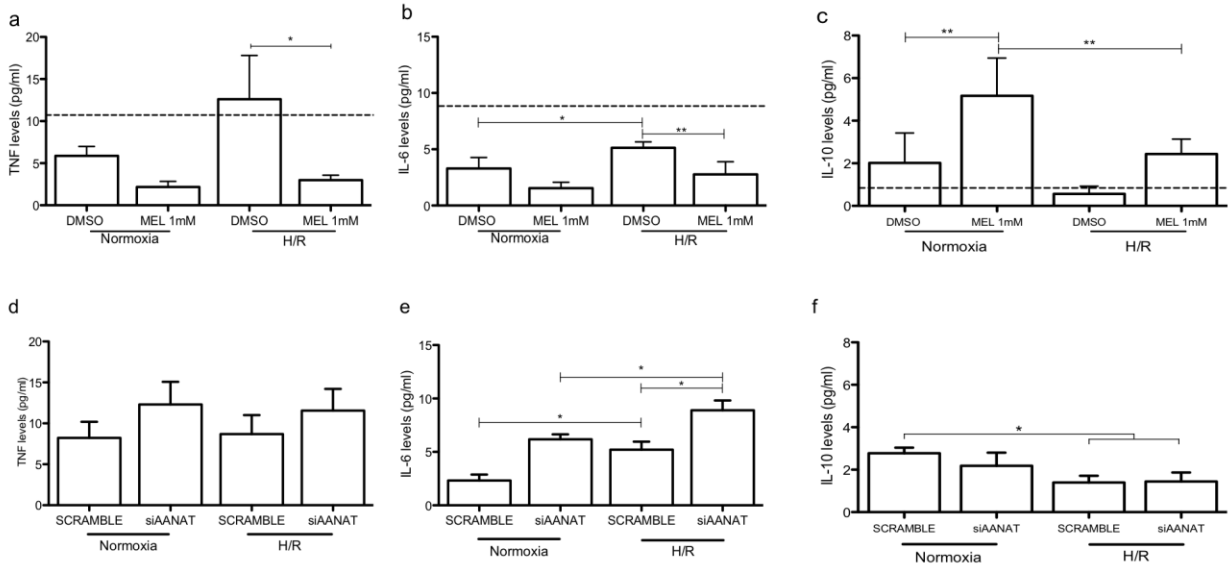

Normoxia + + – –
H/R – – + +
MEL 1 mM – + – +
39 –
IκBα
65 –
NFκBp65
65 –
NFκBp65
(Ser 276)
Total
protein

b


p-NFκBp65 (Ser 276)/total NFκBp65
800
600
400
200
0
***
**
DMSO
MEL
DMSO
MEL
Normoxia
H/R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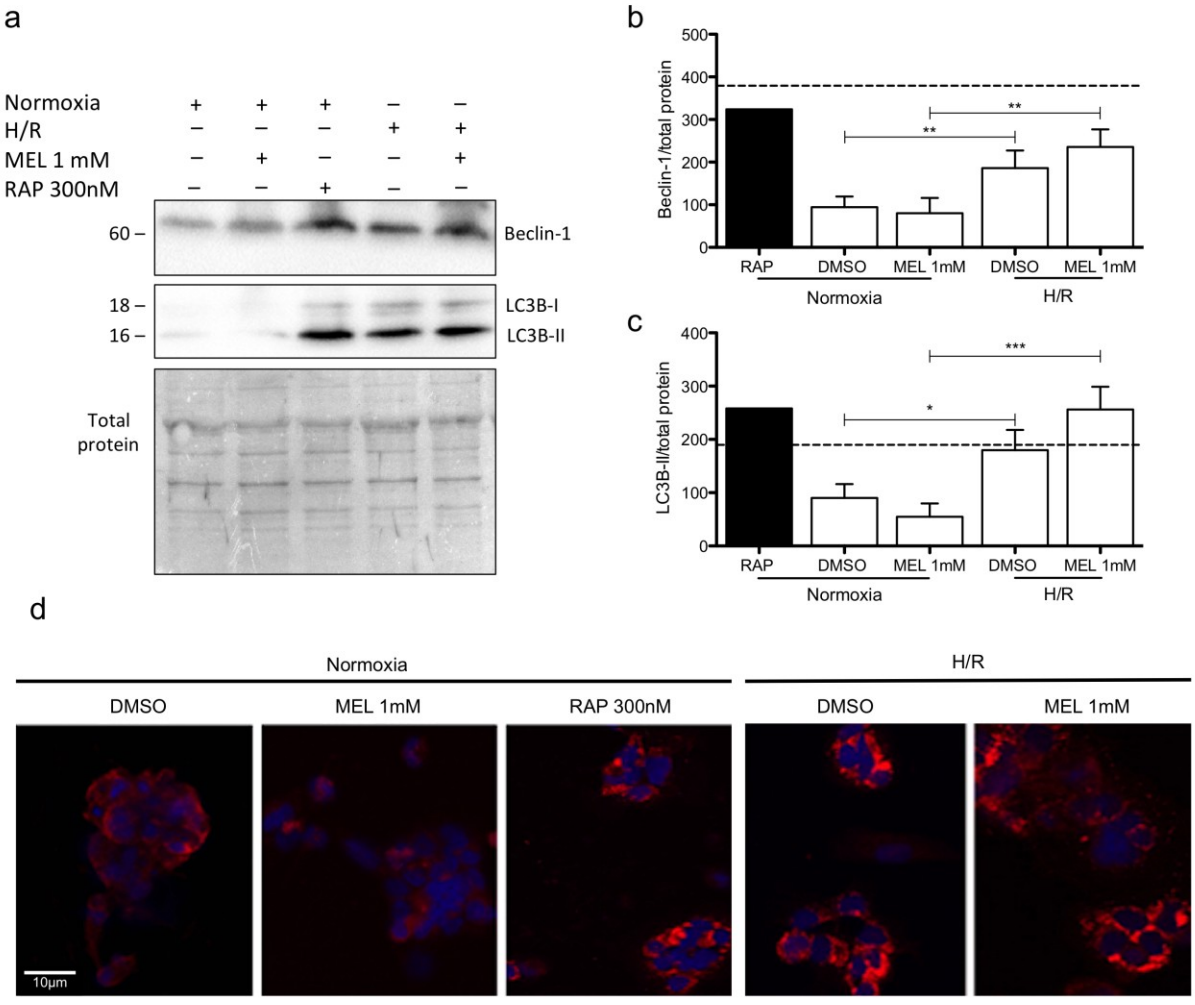
TNF levels (pg/ml)
DMSO
MEL 1mM
DMSO
MEL 1mM
Normoxia
H/R
b
IL-6 levels (pg/ml)
c
IL-10 levels (pg/ml)
d
TNF levels (pg/ml)
SCRAMBLE
siAANAT
e
IL-6 levels (pg/ml)
f
IL-10 levels (pg/ml)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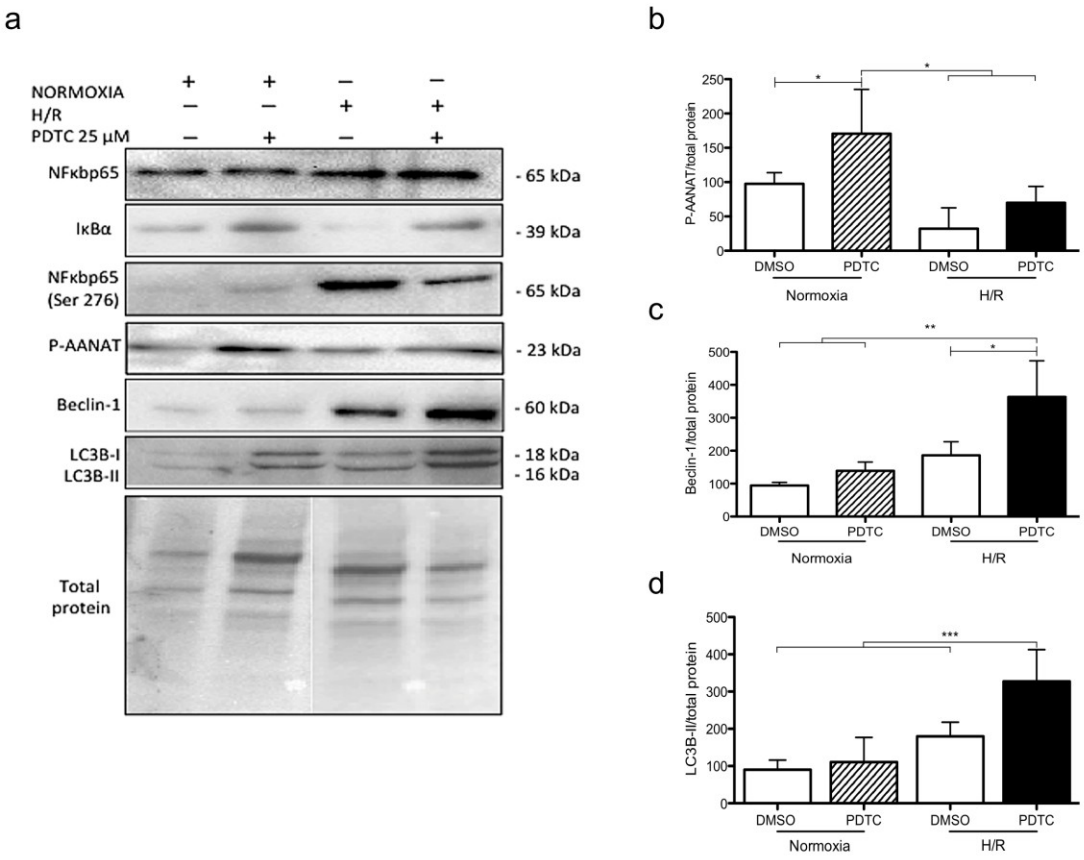
Normoxia + + + – –
H/R – – – + +
MEL 1 mM – + – – +
RAP 300nM – – + – –
60 –
Beclin-1
18 –
16 –
LC3B-I
LC3B-II
Total protein
b
Beclin-1/total protein
500
400
300
200
100
0
**
**
RAP
DMSO
MEL 1mM
DMSO
MEL 1mM
Normoxia
H/R
c
LC3B-II/total protein
400
300
200
100
0
***
*
RAP
DMSO
MEL 1mM
DMSO
MEL 1mM
Normoxia
H/R
d
Normoxia
H/R
DMSO
MEL 1mM
RAP 300nM
DMSO
MEL 1mM
10µm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NORMOXIA
H/R
PDTC 25 μM
NFκbp65
IκBα
NFκbp65 (Ser 276)
P-AANAT
Beclin-1
LC3B-I
LC3B-II
Total protein
- 65 kDa
- 39 kDa
- 65 kDa
- 23 kDa
- 60 kDa
- 18 kDa
- 16 kDa
b
P-AANAT/total protein
DMSO
PDTC
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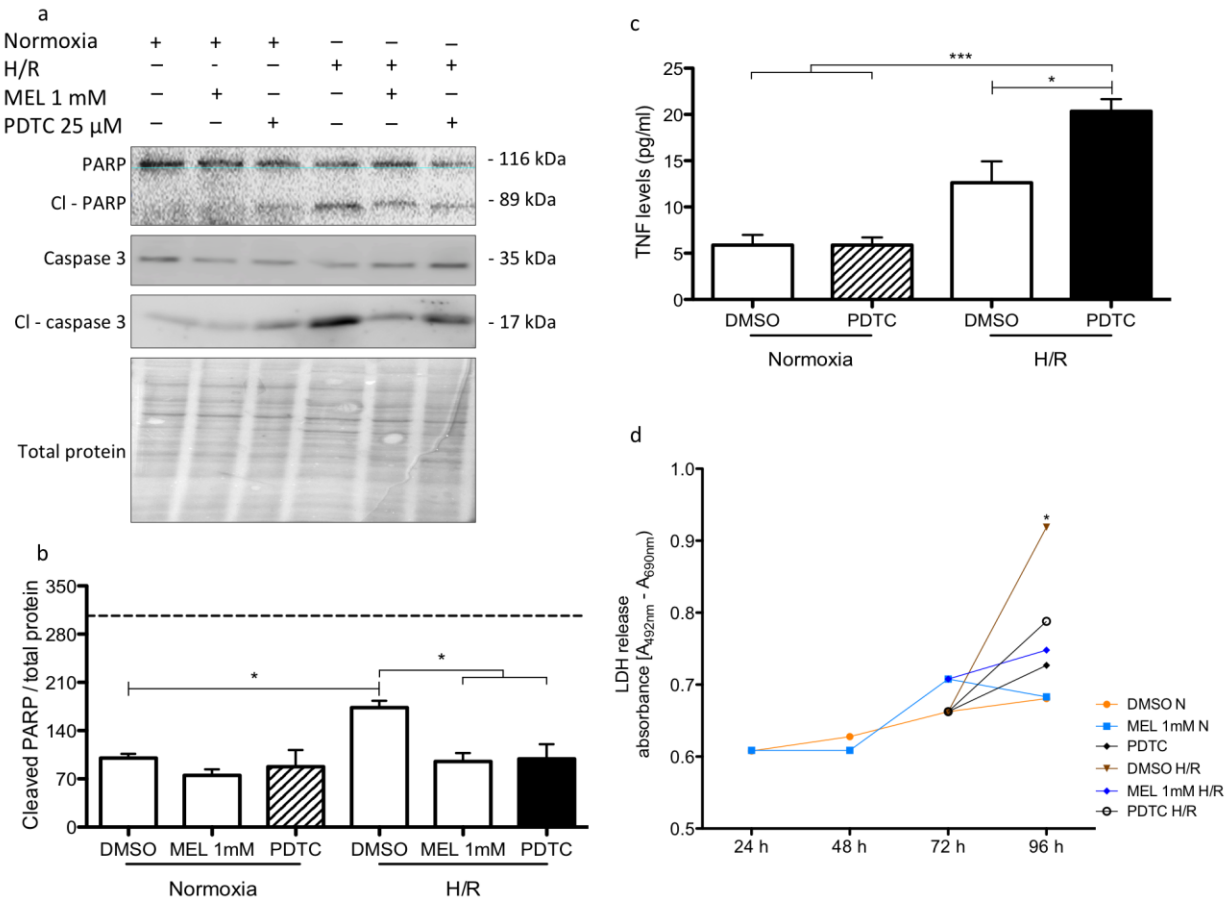
Beclin-1/total protein
d
LC3B-II/total protein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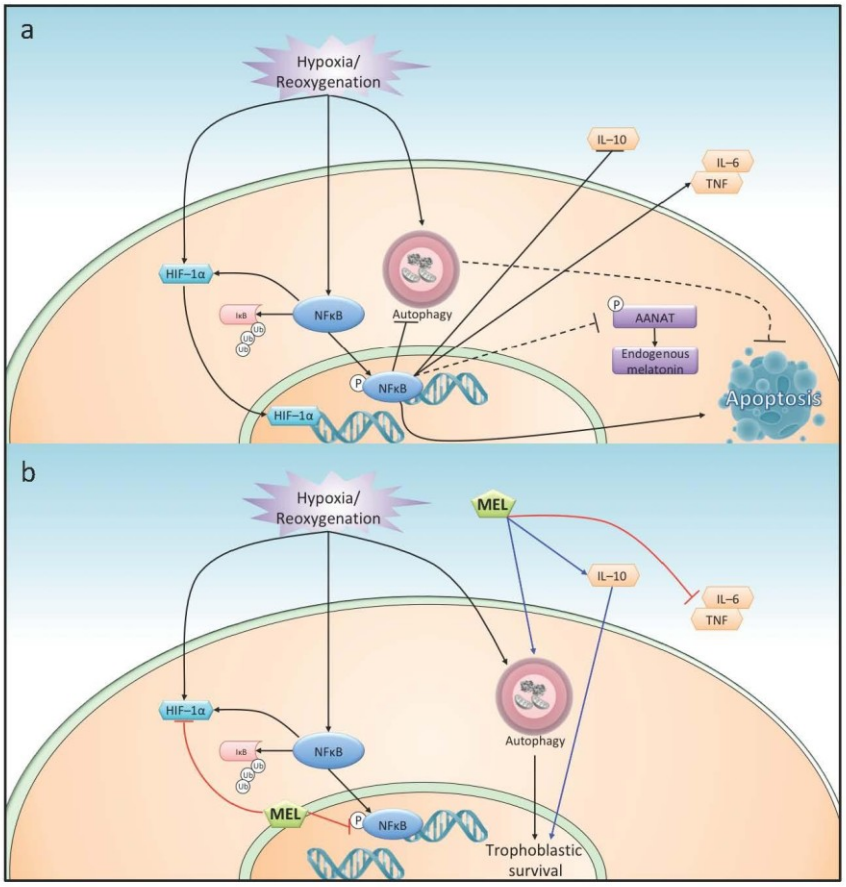
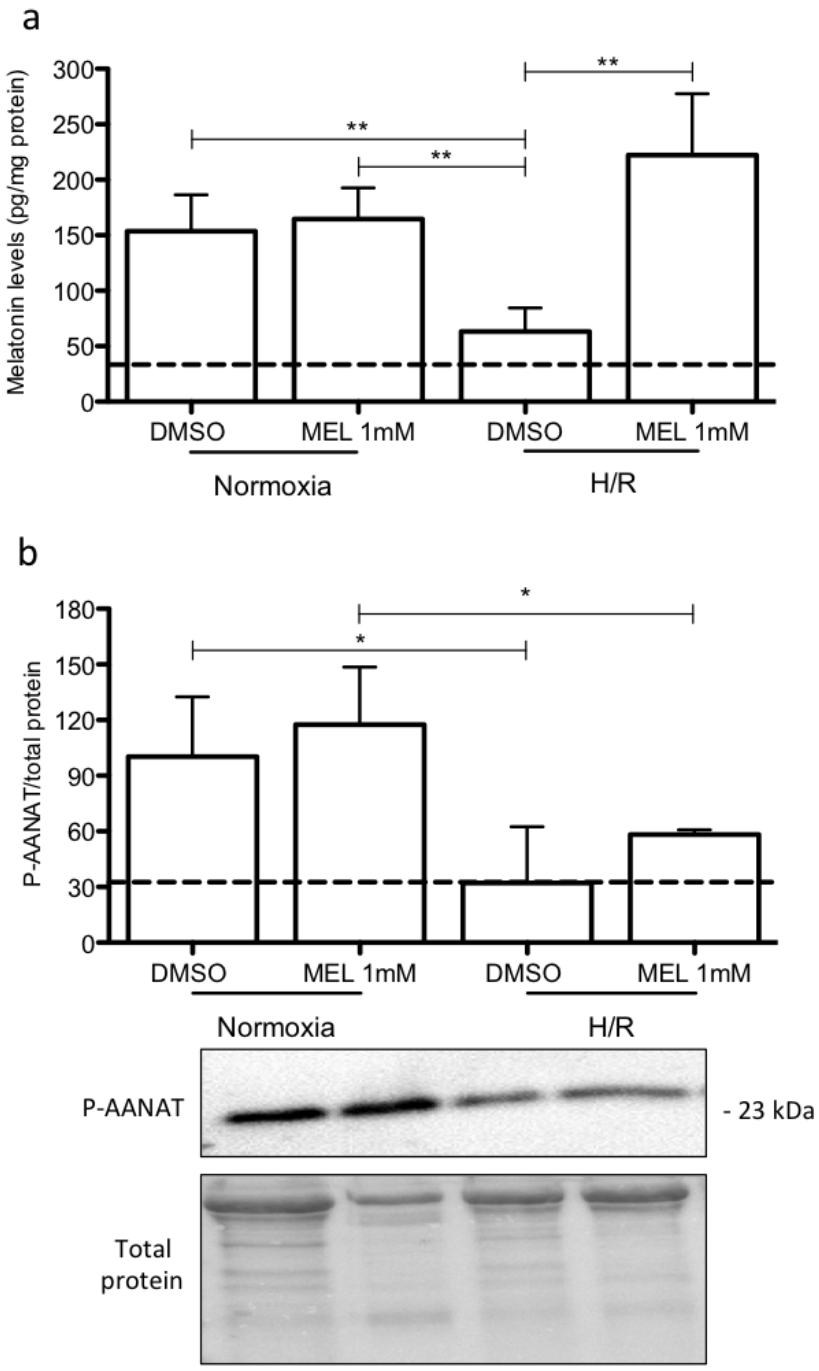
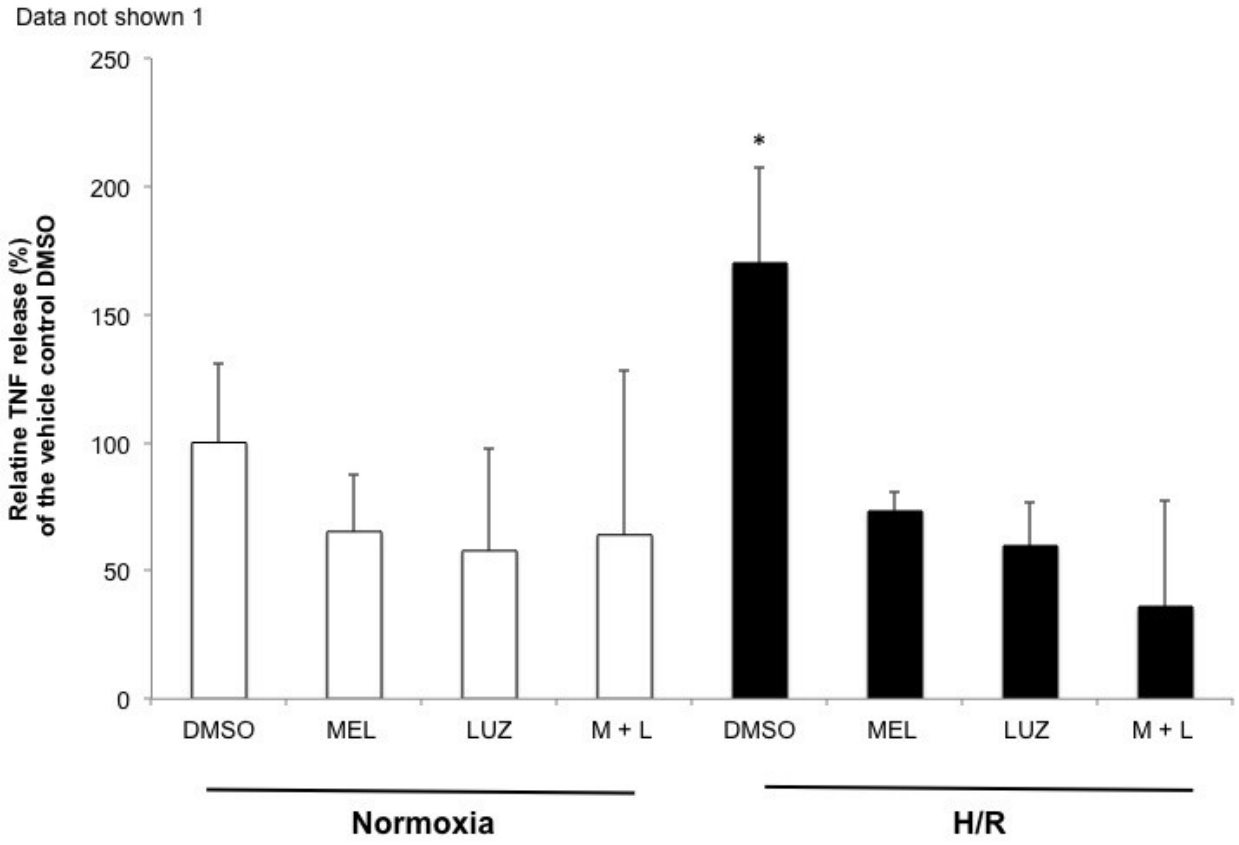
Normoxia + + + – – –
H/R – - – + + +
MEL 1 mM – + – – + –
PDTC 25 µM – – + – – +
PARP
Cl - PARP
Caspase 3
Cl - caspase 3
Total protein
- 116 kDa
- 89 kDa
- 35 kDa
- 17 kDa
b
Cleaved PARP / total protein
350
280
210
140
70
0
DMSO MEL 1mM PDTC DMSO MEL 1mM PDTC
Normoxia
H/R
c
TNF levels (pg/ml)
25
20
15
10
5
0
***
*
DMSO PDTC DMSO PDTC
Normoxia
H/R
d
LDH release
absorbance [A492nm - A690nm]
1.0
0.9
0.8
0.7
0.6
0.5
24 h
48 h
72 h
96 h
DMSO N
MEL 1mM N
PDTC
DMSO H/R
MEL 1mM H/R
PDTC H/R



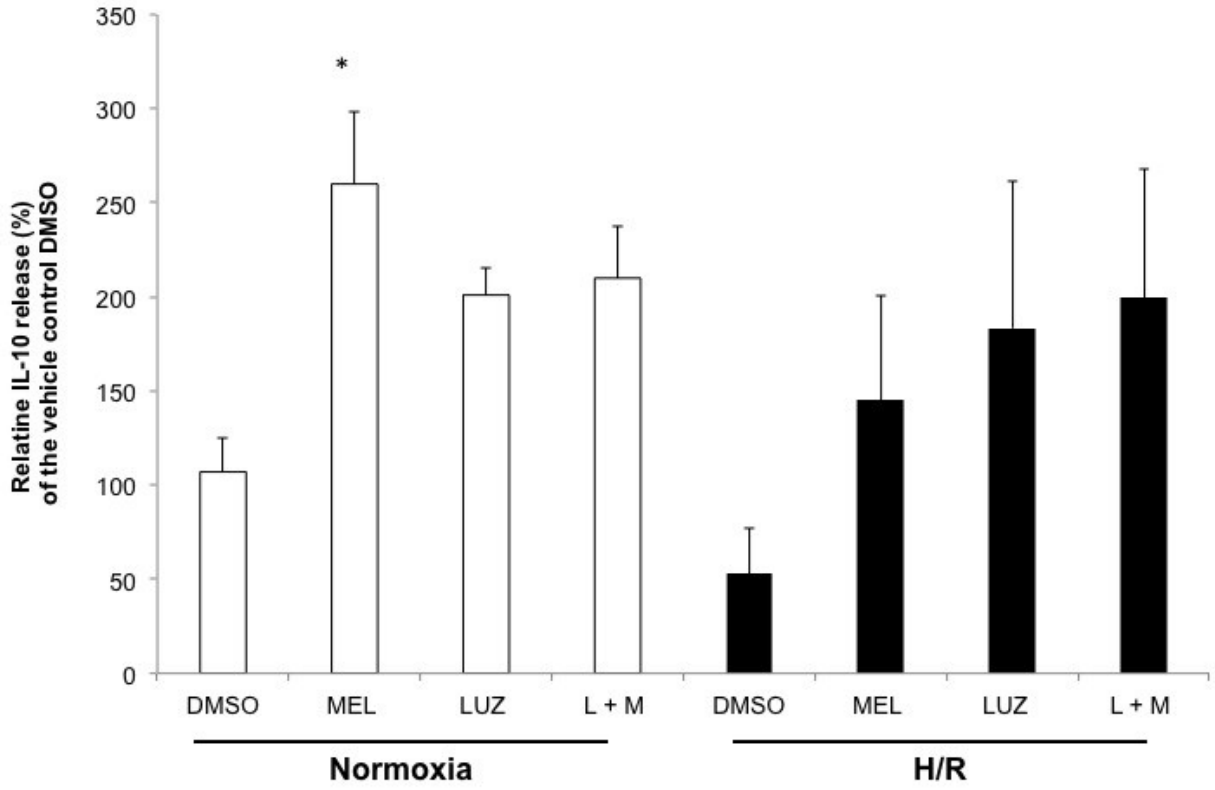

a
Melatonin levels (pg/mg protein)
300
250
200
150
100
50
0
**
**
**
DMSO
MEL 1mM
DMSO
MEL 1mM
Normoxia
H/R
b
P-AANAT/total protein
180
150
120
90
60
30
0
*
*
DMSO
MEL 1mM
DMSO
MEL 1mM
Normoxia
H/R
P-AANAT
- 23 kDa
Total protein

Résultats complémentaires non présentées dans le manuscrit



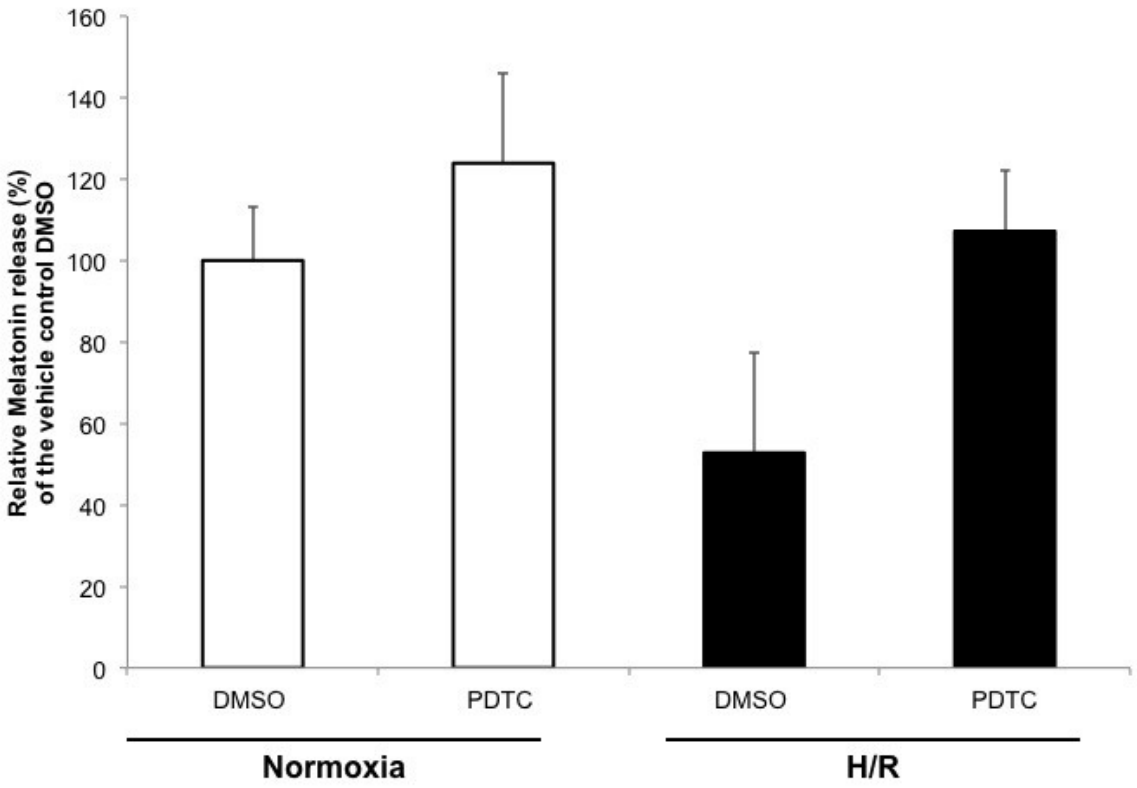
Tumor necrosis factor (TNF) supernatant content was measured using a Multiplex ELISA detection system in villous trophoblast cell-free supernatants after 96 h of culture. Levels of soluble TNF, * $P \leq 0.05$; * $P \leq 0.05$, (N = 4 – 6). Dimethyl sulfoxide ((DMSO) 0.1 %); melatonin (MEL) 1mM ; Luzindole (LUZ 1mM) ; melatonin 1mM and luzindole 1mM (M + L).

Data not shown 2



Interleukin (IL)-10 supernatant content was measured using a Multiplex ELISA detection system in villous trophoblast cell-free supernatants after 96 h of culture. Levels of soluble IL-10. *$P \leq 0.05$ on comparison to DMSO normoxia and melatonin H/R, (N = 4 – 6). Dimethyl sulfoxide ((DMSO) 0.1 %); melatonin (MEL) 1mM ; luzindole (LUZ 1mM) ; melatonin 1mM and luzindole 1mM (M + L).

Data not shown 3



Melatonin supernatant content was measured using an ELISA detection system in villous trophoblast cell-free supernatants after 96 h of culture. (N = 4 – 6). Dimethyl sulfoxide ((DMSO) 0.1 %); Pyrrolidine dithiocarbamate (PDTC) 25 µM.

4. ARTICLE : MELATONIN MODULATES AUTOPHAGY DIFFERENTLY IN TUMOR AND NORMAL PLACENTAL CELLS

Lucas Sagrillo-Fagundes[1,2], Josianne Bienvenue-Pariseault[1,2] and Cathy Vaillancourt[1,2*]

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Contribution de l'étudiant

L'étudiant a participé à l'élaboration de l''étude, réalisé toutes les expériences et analysé tous les résultats. Il a également rédigé l'article et participé au choix du journal de publication.

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Résumé de l'article en français

L'hypoxie/réoxygénation (H/R) induit des dommages oxydatifs et l'apoptose, qui à leurs tour activent l'autophagie, qui dégrade le contenu cellulaire endommagé et induit le *nuclear factor (erythroid-derived 2)-like 2* (Nrf2), et donc l'expression de gènes protecteurs. La mélatonine a des rôles protecteurs dans les cellules normales et des actions cytotoxiques dans les cellules cancéreuses, avec des effets impliquant l'autophagie et la voie du Nrf2. La présente étude montre que la mélatonine module de façon différente les voies d'autophagie et de Nrf2 dans les cellules placentaires normales et tumorales. BeWo, une lignée cellulaire de choriocarcinome placentaire humain et les cytotrophoblastes villeux primaires isolés du placenta à terme normal, ont été maintenus en normoxie (8% O_2) pendant 24 h ou exposés à une hypoxie (0,5% d'O_2 pendant 4 h) suivi de 20 h de normoxie, créant un état d'H/R, en présence ou en absence de 1 mM de mélatonine. La mélatonine a induit une augmentation de 7 fois de l'activation de la protéine 5' adenosine monophosphate-activated protein kinase (AMPK)α, un modulateur en amont de l'autophagie, dans les cellules maintenues en normoxie et de 16 fois en condition d'H/R par rapports au témoin négatif. L'H/R a induit la formation d'autophagosome via l'augmentation des taux de Beclin-1 (de 94%) et d'ATG7 (de 97%). L'H/R a également induit une activité autophagique, indiquée par l'augmentation de 630% des taux de P62 et de Nrf2 de 314%. En condition d'H/R, la mélatonine a réduit l'autophagie de 74% et l'expression de Nrf2 de 66%, conduisant à l'apoptose des cellules BeWo. En revanche, dans les cytotrophoblastes villeux primaires, l'H/R a induit l'activation de l'autophagie et du Nrf2, offrant ainsi une protection contre l'H/R. Cette étude démontre que la mélatonine module de manière différente l'autophagie et la voie du Nrf2 dans les cellules trophoblastiques normales et tumorales, étant cytoprotectrice dans les cellules normales et cytotoxique dans les cellules trophoblastiques tumorales.

Melatonin: The smart molecule that differentially modulates autophagy in tumor and normal placental cells

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SHORT TITLE: Melatonin acts differently in cancer vs normal cells

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Author contributions

All co-authors contributed to the design of the study protocol. LSF and JBP were involved in data acquisition. LSF, was actively involved in the writing of the manuscript assisted by CV. All co-authors provided critical revisions and their final approval for the manuscript's publication in PLOS ONE. This manuscript is not under review elsewhere and all authors are in agreement with the contents of this manuscript.

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Highlights

- H/R induces autophagy and Nrf2 in tumoral and primary trophoblast cells.
- Melatonin inhibits autophagy and Nrf2 under H/R, inducing BeWo cell death.
- Melatonin increases autophagy and Nrf2 under H/R conditions, promoting primary villous trophoblast cells survival.

Abstract

Hypoxia/reoxygenation (H/R) induces oxidative damage and apoptosis. These consequences activate autophagy, which degrades damaged cellular content, as well as inducing the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor, and thereby the expression of protective genes. Melatonin has protective roles in normal cells and cytotoxic actions in cancer cells, with effects involving autophagy and Nrf2 pathways. The current study shows melatonin to differentially modulate autophagy and Nrf2 pathways in tumor and normal placental cells exposed to H/R. BeWo, a human placental choriocarcinoma cell line, and primary villous cytotrophoblasts isolated from normal term placenta, were maintained in normoxia (8% O_2) for 24 h or exposed to hypoxia (0.5% of O_2 for 4 h) followed by 20 h of normoxia, creating a H/R, in the presence or absence of 1 mM melatonin. Melatonin induced a 7-fold increase in the activation of 5' adenosine monophosphate-activated protein kinase (AMPK)α, an upstream modulator of autophagy, rising to a 16-fold increase in cells co-exposed to H/R and melatonin, compared to controls. H/R induced autophagosome formation via the increased expression of Beclin-1 (by 94 %) and ATG7 (by 97%). H/R also induced autophagic activity, indicated by the by the 630% increase in P62, and increased Nrf2 by 314%. In H/R conditions, melatonin reduced autophagy by 74% and Nrf2 expression by 66%, leading to BeWo cell apoptosis. In contrast, in human primary villous cytotrophoblasts, H/R induced autophagy and Nrf2, which melatonin further potentiated, thereby affording protection against H/R. This study demonstrates that melatonin differentially modulates autophagy and the Nrf2 pathway in normal vs. tumor trophoblast cells, being cytoprotective in normal cells whilst increasing apoptosis in tumoral trophoblast cells.

Introduction

Macroautophagy, herein referred to as autophagy, is a highly conserved detoxifying mechanism involving the catabolism of damaged proteins and organelles [1]. Autophagy shows low levels of activity under basal conditions, being inhibited by the cellular sensor, the mechanistic target of rapamycin (mTOR). However, autophagy is activated in suboptimal conditions, such as hypoxia/reoxygenation (H//R) or amino acid starvation (reviewed in [2]). Beclin-1 is an important initiator of autophagy via its activation of the ATG (autophagy-related) proteins. ATG proteins build a double-membrane vesicle, autophagosome, which engulfs cargo to be degraded in lysosomes. The consequent release of simpler structures can restore cellular energy levels and inhibit the deleterious effects of reactive species of oxygen (ROS) [3, 4]. Autophagy upregulates the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2, also called NFE2L2), by the autophagy carrier sequestosome-1/P62 (SQSTM1/P62) [5]. Nrf2 induces defenses against oxidative and other stressors, including by binding to the consensus antioxidant response element (ARE) in their promoters. As with autophagy, Nrf2 is activated in during hypoxia in both normal and cancer cells, including placental cells [6-8].

Alterations in oxygenation are common, reducing cell viability including by increasing ROS and oxidative stress, thereby leading to oxidation and damage of proteins, DNA and lipids [9, 10]. Under such challenge, autophagy is activated leading to increased catabolism of damaged cellular components. BeWo cells, a placental choriocarcinoma model, are frequently utilized to investigate placental physiology, given their ability to synthesize human chorionic gonadotropin (hCG) and their ability to mimic the differentiation of villous cytotrophoblasts (vCTB) into syncytiotrophoblast (STB) [11, 12]. During altered oxygenation, both BeWo and primary trophoblast cells show increased ROS and cell death, thereby inducing autophagic activity, which is modulated by the 5' adenosine monophosphate-activated protein kinase

(AMPK)α and the protein phosphatase 2c (PP2Ac), cellular sensors that are activated to enhance cell survival [13-16].

Melatonin is produced by most cell types, across different tissues and organs. Melatonin is a strong antioxidant, anti-inflammatory and optimizer of mitochondria functioning in non-tumor cells [17, 18]. In contrast, melatonin is cytotoxic in tumor cells, where it has pro-apoptotic and antiproliferative effects [19]. In human placental trophoblastic cells, we have previously shown melatonin to reverse H/R-induced elevations in oxidative stress and cell death, mediated via melatonin effects on inflammation and autophagy [20]. In human choriocarcinoma cells, melatonin disrupts the permeability of the mitochondrial membrane, leading to intrinsic apoptosis [21]. The mechanisms underlying these distinctive effects of melatonin normal vs tumoral placental cells have still to be determined. The comparative effects of melatonin on autophagy and Nrf2 levels in normal vs tumoral placental cells have yet to be investigated. The current study shows that under H/R conditions, the autophagic activity and related pathways are increased in BeWo cells, acting to protect these cells against apoptosis. Melatonin treatment blocks the rise in autophagy in BeWo cells, thereby contributing to their apoptosis. In primary cells, H/R also enhances autophagic activity, which is further increased by melatonin, thereby contributing to cell survival.

Materials and methods

Cell culture

BeWo cells (CCL-98 clone), from American Type Culture Collection (ATCC; Rockville, MD), were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 without phenol red and supplemented with 10% fetal bovine serum (FBS; Hyclone, Tempe, AZ). Human primary vCTB, were obtained from term placentas arising from the spontaneous vaginal delivery of uncomplicated pregnancies after the ethical approval from the CHUM-St-Luc Hospital

(Montreal, QC, Canada) and informed patient consent. vCTB cells were isolated based on the classic method trypsin-DNase/Percoll [22], modified by our group [23]. Briefly, placenta was exposed to four consecutive digestions with trypsin and DNAse. The supernatant of each bath was collected and pooled. The pooled pellets were added to a Percoll gradient, centrifuged, and then the layers containing 40 - 50% of density, which contained mainly villous trophoblast, were collected. Following the isolation, mononuclear villous trophoblasts were immunopurified using the autoMACS™ (Myltenyi Biotec, Santa Barbara), as described previously [24, 25]. All vCTB preparations used in this study were at least of 95% purity after cell sorting. vCTB were cultured in DMEM–High glucose, containing 10% FBS and 1% penicillin-streptomycin (Hyclone).

Cells were plated at the densities of 2.5 x 10^6 cells/ml (BeWo cells) and 3.0 x 10^6 cells/ml (primary vCTB) and promptly put under normoxia (8% O_2, 5% CO_2, 87% N_2) in Modular Incubator Chambers (Billups-Rothenberg, San Diego, CA, USA) for 6 h in order to ensure cell adherence, as previously described. Cells were washed thoroughly with warm media and then maintained under normoxia (8% O_2,) for 24 h or exposed to hypoxic conditions (0.5% O_2,) for 4 h followed by 20 h of normoxia, creating a hypoxia/reoxygenation effect (**Fig. 1a)**. Both BeWo and vCTB cells were treated with 1mM melatonin (Sigma-Aldrich, Oakville, ON, Canada) for 24 h or with its vehicle control, 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Cells were treated for 24 h with Rapamycin ((300 nM) Tocris, Burlington, ON, Canada) and 3-Methyladenine ((5 mM) 3-MA, Tocris), as inducer and inhibitor of autophagy, respectively. Bafilomycin A1 ((10nM) Tocris) was added to cells during the last 2 h of culture to inhibit the fusion of the autophagosome with the lysosome. Sulforaphane ((10μM); Enzo Life Science, New York) was used as an inducer of Nrf2 activity.

MTT assay

Cell viability was determined by monitoring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble form (formazan), by NAD(P)H-

dependent cellular oxidoreductase enzymes. Twenty-four hours after treatment of BeWo cells with DMSO (vehicle control), melatonin 1mM, rapamycin 300 nM or 3-MA 5 mM under normoxia or H/R, 10 µl of MTT, at a final concentration of 5 mg/ml, was added to the media. Four hours after incubation, 100 µl Solubilisation solution (40% (vol/vol) dimethylformamide in 2% (vol/vol) glacial acetic acid) was added to each well to dissolve formazan crystals. After mixing to ensure complete solubilisation, MTT formazan absorbance at 570 nm was measured using with Spectra Max M5 (Molecular Devices). Results are presented as a percent of vehicle control (DMSO).

hCG secretion

To evaluate the secretion of hCG by BeWo cells exposed to vehicle control (DMSO), melatonin 1 mM, rapamycin 300 nM or 3-MA 5 mM under normoxia or H/R, cell culture media were collected and centrifuged, after which supernatants were stored at −20°C until assayed. The secretion of hCG culture was evaluated by enzyme-linked immunosorbent (ELISA) assay according to manufacturer's instructions (IBL International; Toronto, ON, Canada). Results are presented as a percent of vehicle control.

Immunoblotting

To analyze protein expression, BeWo cells and primary vCTB were rinsed with PBS and lysed with ice-cold modified radioimmunoprecipitation (RIPA) buffer (50 mmol/l Tris-HCl pH 7.4, 1% NP-40, 0,25% Na-deoxycholate, 150 mmol/l NaCl and 1 mmol/l EDTA) containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Waltham, MA). Twenty µg of protein were separated on Mini-PROTEAN® TGX™ Precast Protein Gels (4–15% precast polyacrylamide gel) (Bio-Rad, Saint-Laurent, QC, Canada), followed by transfer to

Polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Membranes were then incubated with antibodies as described in **Table 1**. Blots were developed with enhanced chemiluminescence reagent (Bio-Rad). Protein levels were expressed as a ratio of a specific band density and total protein stained using Pierce™ Reversible Protein Stain Kit (Pierce Biotechnology), as previously described [26, 27]. Bands were quantified using Image Lab software 6.0 (Bio-Rad).

189 Table 1: Antibodies used for Western blot analyses.

Antibody	Dilution	Incubation	Source
Primary antibodies			
Phospho-AMPKα (T183)	1:500	O/N, 4 °C	R&D Systems (AF2509)
AMPKα	1:1000	O/N, 4 °C	R&D systems (AF3197)
Phospho-PP2Ac (Tyr 307)	1:300	O/N, 4 °C	R&D systems (AF3989)
PP2Ac	1:2000	O/N, 4 °C	Millipore (05-421)
Beclin-1	1:1000	O/N, 4 °C	Cell Signaling (3495)
ATG 5	1:1000	O/N, 4 °C	Cell Signaling (12994)
ATG16	1:1000	O/N, 4 °C	Cell Signaling (8089)
ATG7	1:1000	O/N, 4 °C	Cell Signaling (8558)
ATG12	1:1000	O/N, 4 °C	Cell Signaling (4180)
LC3-B	1:2000	1 h, 4 °C	Sigma-Aldrich (L7543)
TOM20	1:2000	O/N, 4 °C	Santa Cruz (FL-145)
Nrf2	1:2000	O/N, 4 °C	Santa Cruz (SC-722)
PARP-1	1:1000	O/N, 4 °C	Cell Signaling (9542)
Cleaved PARP-1	1:1000	O/N, 4 °C	Cell Signaling (5625)
Histone H3	1:500	O/N, 4 °C	ABCAM (ab1791)
α-tubulin	1:5000	O/N, 4 °C	Cell Signaling (2144)
Secondary antibodies			
Anti-goat-HRP	1:10 000	1 h, RT	Millipore (AP180P)
Anti-mouse-HRP	1:10 000	1 h, RT	Millipore (AP192P)
Anti-rabbit-HRP	1:10 000	1 h, RT	Millipore (AP182P)

190 AMPKα : 5' AMP-activated protein kinase α; PP2Ac: Protein Phosphatase 2Ac; ATG:
191 Autophagy gene; LC3-B: Microtubule-associated proteins 1A/1B light chain 3B; TOM20:
192 Translocase of the outer membrane; Nrf2: Nuclear factor erythroid 2–related factor 2; PARP-1:
193 Poly (ADP-Ribose) Polymerase

194

195

196

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Immunofluorescence

BeWo cells and primary vCTB were fixed with methanol at – 20 °C for 20 min. Cells were incubated with 2% FBS in PBS for 1 h to eliminate nonspecific antigen binding. BeWo cells were stained overnight (4 °C) for LC3B (1:150) or cytochrome c oxidase iv (cox iv) (1:300). vCTB were stained overnight (4 °C) for LC3-B (1:150) and Nrf2 (1:150). After the incubation with the primary antibodies, both cell types, were then incubated with anti-mouse or anti-rabbit conjugated to Alexa Fluor fluorescent dye. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (dapi) present in the antifade reagent, prolong™ gold (Thermo Fisher scientific). Immunofluorescence was analyzed at 400 x using a Leica DMRE fluorescence microscope (Ceerfield, il) with a Cooke sensicam high-performance digital CCD camera (Romulus, mi).

Nucleus isolation

BeWo cells (2.0 x 10^6 cells) were harvested with TrypLE and had the nuclei isolated following the guidelines of the isolation kit, NE-PER Nuclear and Cytoplasmic Extraction (Thermo Fisher Scientific). The protein yield of the nuclear and the cytosolic extracts was measured with the BCA protein assay. The purity of the nuclear and the cytosolic extracts were validated with the western blotting of the protein Histone H3 (nuclear fraction) and α-tubulin (cytosolic fraction).

Statistical analysis

All data represent at least four different BeWo cell passages or three primary vCTB cultures. Statistically significant differences ($p \leq 0.05$) of parametric results were determined by Student's *t*-test or one-way analysis of variance (ANOVA) and identified by the *post hoc* test of Student-Newman-Keuls. Data were analyzed using GraphPad Prism (version 5.04).

Results

Melatonin increases cellular disruption caused by H/R in BeWo cells

As shown in **figure 1b**, BeWo cells exposed to H/R showed a 1.38-fold reduction in viability, compared to normoxia. BeWo cells exposed to H/R and concomitantly with melatonin or 3-MA, an inhibitor of autophagy, also showed lower cell viability (reduction of 1.29 and 1.44-fold, respectively), compared to controls under normoxia. Importantly, in comparison to BeWo cells exposed only to H/R, cells exposed to H/R and melatonin showed significantly lower viability (reduction of 1.19-fold). Cells exposed to H/R and rapamycin, which induces autophagy, showed no differences in cell viability compared to normoxia, suggesting that rapamycin may improve cell viability in BeWo cells exposed to the suboptimal oxygen. In BeWo cells under normoxic conditions, melatonin led to a 1.28-fold reduction in the release of β-hCG, vs DMSO controls (**Fig. 1c**). H/R decreased the release of β-hCG in BeWo cells exposed to vehicle control by 1.38-fold and by 2.1-fold in cells exposed to 3-MA (**Fig. 1c**). In BeWo cells exposed to rapamycin the release of β-hCG was not affected, independently of the levels of oxygenation. The similarity of the viability and β-hCG results in cells treated with 3-MA may suggest that the absence of autophagy affects the global cell viability of BeWo cells exposed to H/R.

Fig. 1: Melatonin decreases the viability of BeWo cells under hypoxia/reoxygenation.

(a) BeWo cells were exposed to normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia (4 h) /reoxygenation (20 h) (H/R) (0.5% O_2; 5% CO_2; 94.5% N_2). Cells were treated or, not (DMSO), with 1 mM melatonin (MEL). Rapamycin (RAP) (300 nM) was used to induce of autophagy. 3-methyladenine (3-MA) (5mM) and bafilomycin A1 (BAF A1) (10 nM) were used as inhibitors of

248 phagosome formation and phagosome-lysosome merger, respectively. Cells were treated with the
249 indicated concentrations of MEL or RAP 24 h. (b) Cell viability was measured using the MTT
250 assay and demonstrated as % of control (DMSO normoxia) (c) and the release of β-hCG in the
251 cell media was measured by ELISA and demonstrated as % of control (DMSO normoxia). Data
252 shown are mean ± SD and were analyzed using Student's *t*-test (* $p<0.05$ and ** $p<0.01$,
253 compared to the same treatment in normoxic conditions; whilst $^{\#}p<0.05$ indicates comparison to
254 vehicle control, DMSO), n=7.
255
256
257 To further investigate the mechanisms involved in the cell viability disruption caused by
258 melatonin in challenging conditions such as H/R, we further investigated the level of
259 phosphorylation of the enzyme AMPKα (Thr 172). The phosphorylation of AMPKα is mainly
260 induced by lower ATP levels [28] or, in cases of hypoxia, by ROS [15]. The phosphorylation of
261 AMPKα was normalised to the total levels of AMPKα, which were steady, irrespective of
262 conditions and treatments. In BeWo cells, phospho-AMPKα levels were increased 6.7 fold in
263 cells exposed to 1 mM melatonin in normoxia, compared to vehicle controls. In BeWo cells
264 exposed to H/R without melatonin, phospho-AMPKα levels were increased 12-fold, compared to
265 vehicle controls. The maximal activation of AMPKα was achieved by melatonin under H/R
266 conditions, where phospho-AMPKα levels were increased 16-fold, compared to all other
267 conditions (**Fig. 2a**). In addition, we investigated the activation of the factor PP2Ac, which has a
268 protective role against hypoxia [29]. The activation of autophagy is among the protective actions
269 driven by the dephosphorylation at Tyr 307 of PP2Ac, which is considered a marker of poor
270 prognosis in malignant cells [13]. Interestingly, BeWo cells show lower levels of phosphorylated
271 PP2Ac when exposed to H/R, independently of melatonin treatment (**Fig. 2b**), compared to
272 normoxia. Beclin-1 is considered a molecular scaffold of autophagy modulators (including
273 AMPKα and PP2Ac) and controls autophagosome formation. Beclin-1 protein levels were

increased in BeWo cells exposed to H/R, compared to normoxia. Melatonin did not modulate Beclin-1 levels in either normoxia or H/R conditions. As expected, H/R upregulates upstream regulators of autophagy, with autophagy being inhibited by melatonin in BeWo cells.

Fig 2. Hypoxia/reoxygenation (H/R) and melatonin modulate upstream autophagic factors in BeWo cells. (a) Relative optical density of phospho-AMPKα (Thr 172), normalized with total AMPKα. The relative molecular mass (kDa) is indicated to the right of the representative blot. (b) Relative optical density of phospho-PP2Ac (Tyr 307), normalized with total PP2Ac. (c) Relative optical density of Beclin-1 with total protein. Total protein amounts were measured by staining with MemCode reversible protein stain. Data are shown as mean ± SD and were analyzed using ANOVA, followed by Student-Newman-Keuls (*p<0.05; **p<0.01; ***p<0.001), n = 5.

Melatonin regulates autophagy in BeWo cells

To determine the downstream pathway involved in the elongation of the autophagosome, we investigated the protein levels of ATG5-ATG12, ATG 16β, ATG 16α, ATG7, and the lipidation of LC3B. The ATG5-ATG12 complex is responsible for the activation (*i.e.* lipidation) of LC3B [30], which is anchored in the autophagosome complex by ATG16 [31]. Rapamycin, which was used as a positive control of autophagy, as expected, increased ATG5-ATG12, ATG16, LC3B-II, and ATG7 under normoxia and H/R conditions, compared to vehicle controls. As with rapamycin, both ATG16 and ATG5-ATG12 protein levels were increased by H/R. Melatonin had no effect on ATG5-ATG12 protein levels, whereas it increased ATG16 in H/R (**Fig. 3a**). ATG7 controls the activation of the whole formation of the autophagosome [2]. ATG7

was significantly increased (by 1.97-fold) in BeWo cells exposed to H/R, in comparison to normoxia. Melatonin increased the ATG7 content by 1.56-fold under normoxia. In H/R conditions, melatonin led to a significant 1.24-fold increase in ATG7 levels, compared to BeWo exposed to DMSO (**Fig. 3a and b**). The activation of LC3B into LC3B-II was significantly increased in BeWo cells exposed to H/R compared to normoxia, with melatonin having no significant effect (**Fig. 3a and 3c**). The lipidation of LC3B is visually characterized by the formation of different puncta, compared to non-active LC3B, which presents with a more uniform cytosolic distribution. As showed in the **figure 3d,** rapamycin and H/R activate the formation of LC3b-puncta, which may indicate the presence of an active autophagosome. Melatonin is not able to significantly activate the whole machinery responsible for the autophagosome formation.

Fig. 3: Hypoxia/reoxygenation and melatonin differentially regulate autophagosome formation in BeWo cells. (a) BeWo cells were exposed to normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia (4 h) /reoxygenation (20 h) (H/R) (0.5% O_2; 5% CO_2; 94.5% N_2) with or without treatment with melatonin (1 mM) or rapamycin (300 nM). Protein levels of autophagy-related (ATG) ATG5-ATG12, ATG16β, ATG16α, and ATG7 were detected by immunoblotting using monoclonal antibodies. Microtubule-associated proteins 1A/1B lightchain 3B (LC3B) was detected by immunoblotting using a polyclonal antibody. The relative molecular mass (kDa) is indicated to the right of the representative blot. Total protein amounts were measured by staining with MemCode reversible protein stain. ATG16β and ATG16α were represented with two distinct blot images, separated by a dotted line. (b) Relative optical density of ATG7 normalized with total protein is expressed in comparison to vehicle control (DMSO) in normoxia. (c) Relative optical density of lipidated LC3B (LC3B-II) were normalized with total protein being

expressed in comparison to vehicle control (DMSO) in normoxia. Data shown are mean ± SD and were analyzed using ANOVA, followed by Student-Newman-Keuls (*p<0.05; **p<0.01; ***p<0.001 compared to the same treatment in normoxic conditions, $^{\#}$ p<0.05: indicates significance in comparison to DMSO in H/R), n = 5. (d) The cells were fixed and probed for LC3B using immunofluorescence staining, n=4. BeWo cells cultured under normoxia or H/R treated with dimethyl sulfoxide (DMSO) 0.1%, melatonin 1mM or rapamycin 300 nM were fixed in methanol, stained for LC3B (red), counterstained with 4',6-diamidino-2-phenylindole (DAPI) (nucleus staining blue), and observed by confocal microscopy at 20x magnification.). Scale bar represents 10 μm.

Melatonin reduces autophagic activity and activation of Nrf2 in BeWo cells exposed to H/R.

To further investigate the role of melatonin in the regulation of the autophagy pathway, bafilomycin A1 (BAF A1) was added to the BeWo cell medium during the last 2 h of cell culture under H/R. BAF A1 prevents the fusion of the autophagosome with the lysosome, thereby allowing the investigation of autophagic activity by measuring the protein levels of SQSTM1/P62, which acts as an adaptor for protein trafficking, aggregation, and degradation [32]. **Figure 4a** indicates that, in H/R conditions, BAF A1 increased SQSTM1/P62 by 6.3 fold, compared to BeWo cells under H/R without BAF A1 treatment, suggesting a high level of autophagic activity. In contrast, the BeWo cells exposed to H/R that were treated with melatonin and BAF A1 showed significantly lower levels of SQSTM1/P62, vs cells treated with vehicle control (DMSO), indicating lower autophagic activity. The dashed line represents the protein levels of SQSTM1/P62 in BeWo cells exposed to H/R and treated with rapamycin, as a positive control of the degradation of the autophagic cargo, including SQSTM1/P62. SQSTM1/P62

shares a positive feedback loop with the transcription factor Nrf2 [5], with Nrf2 showing significantly higher protein levels in BeWo cells exposed to H/R compared to normoxia, with Nrf2 levels similar to that of cells exposed to the positive control, sulforaphane, an Nrf2 activator (**Fig. 4b**). BeWo cells exposed to H/R and treated with melatonin, showed significantly less Nrf2 protein levels compared to DMSO, suggesting that melatonin downregulates the Nrf2 protective pathway in these cells. We therefore investigated the Nrf2 nuclear content, which represents the active form of this transcription factor, and found similar results: cells exposed to H/R plus melatonin showed 3-fold reduction in nuclear Nrf2, compared to cells exposed to H/R without melatonin treatment (**Fig. 4d**). As previously described [21], melatonin induces apoptosis in BeWo cells (**Fig. 4c**). The cleavage of poly (ADP-ribose) polymerase (PARP), a hallmark of apoptosis, was increased in BeWo cells exposed to H/R, compared to normoxia. Moreover melatonin in normoxia increased the cleavage of PARP in comparison to the control vehicle. In contrast, cells exposed to the positive control of autophagy, rapamycin, in both normoxia and H/R showed decreased levels of cleaved PARP, suggesting that the induction of autophagy has a beneficial effect on BeWo cell survival, and that the downregulation of autophagy by melatonin contributes to BeWo cell death.

Fig. 4: Melatonin reduces autophagy turnover thereby increasing BeWo cell vulnerability to H/R. BeWo cells were exposed to normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia (4 h) /reoxygenation (20 h) (H/R) (0.5% O_2; 5% CO_2; 94.5% N_2). (a) Sequestosome-1, SQSTM1/P62, protein expression was detected by immunoblotting using a polyclonal antibody in BeWo cells exposed to H/R. Cells were treated with dimethyl sulfoxide (DMSO) 0.1% or 1 mM melatonin (MEL) for 24 h. Cells were also treated with BAF A1 (10 nM) during the last 2 h of BeWo cell culture. (b) Nrf2 protein expression was detected by immunoblotting using a polyclonal antibody in BeWo cells exposed to normoxia or H/R. Sulforaphane 10 μM was added to BeWo cells for

24 h as an Nrf2 inducer. (c) Relative optical density of the carboxy-terminal catalytic domain of the poly (ADP-ribose) polymerase (PARP), which was cleaved between the Asp214 and Gly215 was normalized with total PARP. Both cleaved PARP and PARP1 were detected by immunoblotting using a polyclonal antibody. (d) The nuclear and the cytosolic contents of the Nrf2 protein of BeWo cells exposed to H/R were detected by immunoblotting. Histone H3 and α-tubulin were used as protein markers of the purity of nuclear and cytosolic fractions, respectively. Representative blots are shown below the graphs and the relative molecular mass (kDa) is indicated to the right of the blot. Total protein amounts were measured by staining with MemCode reversible protein stain. Data shown are mean ±SD and were analyzed using ANOVA, followed by Student-Newman-Keuls (a, b, c) or Student's *t*-test (d) (*p<0.05; **p<0.01; ***p<0.001), n = 4-5.

Melatonin and hypoxia/reoxygenation decrease the mitochondrial content in BeWo cells

Previous work indicates that melatonin reduces the mitochondrial membrane potential in BeWo cells [21], with mitochondrial content being reduced under hypoxic conditions [33]. In normoxia conditions, melatonin led to a 1.8-fold decrease in the mitochondrial marker TOMM20. As demonstrated in the **figure 5a**, cells exposed to H/R show a 2.5-fold decrease in TOMM20 protein level, compared to normoxia. In H/R conditions, melatonin led to a 4.5-fold decrease in TOMM20, which was significantly lower compared to all other conditions. BAF A1 was added in H/R to block the merging of the autophagosome with lysosome and the consequent catabolism of damaged cellular components. BAF A1 was therefore used as a control to investigate if mitophagy is involved in the reduction of the TOMM20 mitochondrial content under H/R conditions. As demonstrated in the **figures 5a and 5b**, BeWo cells exposed to H/R and treated with BAF A1 presented similar lower mitochondria content in comparison to the

normoxia control, suggesting that autophagy was responsible for the reduction of the mitochondrial content observed under H/R. **Figure 5b** shows the colocalization of the mitochondrial marker COX IV and LC3B in BeWo cells. In H/R, cells exposed to melatonin or BAF A1 presented a similar pattern of colocalization of COX IV and LC3B. This may indicate that the decreased mitochondrial content is due to mitophagy in BeWo cells exposed to H/R and treated with melatonin, vs H/R exposure without melatonin.

Fig. 5: Melatonin and hypoxia/reoxygenation decrease the mitochondrial content in BeWo cells. BeWo cells were exposed to normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia (4 h) /reoxygenation (20 h) (H/R) (0.5% O_2; 5% CO_2; 94.5% N_2) with or without treatment with melatonin (1 mM) or bafilomycin (BAF A1: 10 nM). (a) TOMM20 protein level was detected by immunoblotting using a polyclonal antibody. The relative molecular mass (kDa) is indicated to the right of the representative blot. Total protein amounts were measured by staining with MemCode reversible protein stain. Data shown are mean ± SD and were analyzed using ANOVA, followed by Student-Newman-Keuls (*p<0.05, compared to DMSO in normoxia; $^{\#}p$<0.05, when compared to all other groups), n=4. (b) BeWo cells cultured in H/R conditions and were fixed and probed for cytochrome c oxidase IV (COX IV) and microtubule-associated proteins 1A/1B light chain 3B (LC3B) using immunofluorescence staining. Representative pictures are shown for both COX IV + LC3B staining, COX IV + DAPI staining, and LC3B staining + DAPI. Scale bar represents 10 μm.

In H/R, melatonin upregulates autophagy in primary vCTBs

As shown in the **figure 6a**, primary vCTBs exposed to H/R with or without treatment with melatonin, had raised protein level of Beclin-1 and LC3B lipidation, compared to normoxia. Intriguingly, in H/R, melatonin treatment increases dramatically the LC3B lipidation (**Fig. 6a, d**). In H/R, primary vCTBs treated with BAF A1, vs not, showed increased SQSTM1/P62

426 protein levels, suggesting that the autophagic turnover was activated. The utilization of BAF A1
427 showed melatonin to have an additive effect on autophagic turnover (**Fig. 6b**). Nrf2 was also
428 significantly increased (by 2.25-fold) in primary vCTBs exposed to H/R, compared to normoxia
429 (**Fig. 6a, c, d**). Following melatonin treatment, Nrf2 expression was further upregulated (by
430 1.44-fold), vs H/R only exposure. Moreover, melatonin led to the nuclear translocation of Nrf2
431 and to the lipidation of LC3B, as shown in the **figure 6d**. Both the increased lipidation of LC3B
432 and the nuclear translocation of Nrf2 indicate a protective role of melatonin in human vCTB
433 under H/R.
434
435
436 **Fig. 6: Melatonin increases autophagy turnover and Nrf2 expression in primary villous**
437 **cytotrophoblasts exposed to hypoxia/reoxygenation.** Human primary villous cytotrophoblasts
438 (vCTBs) were exposed to normoxia (8% O_2; 5% CO_2; 87% N_2) or hypoxia (4 h) /reoxygenation
439 (20 h) (H/R) (0.5% O_2; 5% CO_2; 94.5% N_2). Cells were treated with dimethyl sulfoxide (DMSO)
440 0.1% or 1 mM melatonin (MEL) for 24 h. (a) Protein expression of nuclear factor (erythroid-
441 derived 2)-like 2 (Nrf2), Beclin-1, and microtubule-associated proteins 1A/1B lightchain 3B
442 (LC3B) were detected by immunoblotting. (b) Sequestosome-1 is also known as the ubiquitin-
443 binding protein p62 (SQSTM1/P62), and its protein expression was detected by immunoblotting
444 using a polyclonal antibody in vCTB exposed to H/R. Cells were treated with dimethyl sulfoxide
445 (DMSO) 0.1% or 1 mM melatonin (MEL) for 24 h. vCTB were also treated with bafilomycin A1
446 (10 nM) during the last 2 h of culture. Total protein amounts were measured by staining with
447 MemCode reversible protein stain. The relative molecular mass (kDa) is indicated to the right of
448 the representative blot. (c) Relative optical density of Nrf2 normalized with total protein. Data
449 shown are mean ± SD and were analyzed using ANOVA, followed by Student-Newman-Keuls
450 (*p<0.05; ***p<0.001), n = 4. (d) vCTB cells were fixed and probed for Nrf2 (green) and LC3B

(red) using immunofluorescence staining. 4',6-diamidino-2-phenylindole (DAPI; blue) was applied as a nucleus stain. Scale bar represents 10 μm.

Discussion

H/R disturbs the cellular homeostasis; leading to the activation of survival mechanisms, such as autophagy and the transcription factor Nrf2 [6, 34]. Most data indicates melatonin to be protective to normal cells and cytotoxic to cancer cells [19, 35]. In accord with these studies, the present study shows that H/R is able to activate both autophagy and Nrf2 in normal and tumoral trophoblasts, with melatonin inhibiting autophagy and Nrf2 in the BeWo choriocarcinoma cell line BeWo, whilst upregulating these pathways in human primary vCTBs. The above results demonstrate, for the first time, that autophagy is activated by H/R, but inhibited when treated with concomitant melatonin treatment in BeWo cells. Despite an increased total and nuclear protein fraction in BeWo cells exposed to H/R, Nrf2 activation is inhibited by melatonin resulting in the activation of cell death via the cleavage of PARP-1. In contrast, human primary vCTBs exposed to H/R and treated with melatonin showed increased autophagy and Nrf2 activity, which corroborates previous data showing a protective role of melatonin for normal placental cells [20, 36].

Previous work has shown melatonin to be produced, and to have important protective functions, in primary human trophoblasts as well as in the pregnancy [20, 21, 36, 37]. H/R increases oxidative stress levels and lowers primary villous trophoblast viability, with melatonin affording protection against such H/R effects ([20]; Fagundes, et al. *in press*). In the current study, BeWo cells exposed to H/R showed reduced cell viability and lower hCG release (a marker of the endocrine function of normal and tumoral placental trophoblasts), compared to normoxia [38]. The treatments with melatonin or 3-MA, an inhibitor of autophagy, were not able to rescue the levels of viability or hCG, whereas rapamycin, which is largely used as an activator

476 of autophagy via mTOR inhibition, rescued the viability of BeWo cells exposed to H/R.
477 Importantly, the current results show that the upstream modulators of autophagy, PP2Ac and
478 AMPKα, are activated under H/R conditions. Such data on cell viability and rapamycin effects
479 implicate autophagy in the survival of BeWo choriocarcinoma cells challenged by H/R.
480 Intriguingly, the activation of LC3B in its lipidated form, LC3B-II, was not significantly
481 modulated by H/R in BeWo cells. This could be due to the high basal activity of autophagy
482 already described in cancer cell models exposed to hypoxic conditions, where results indicate
483 increased activation of LC3B only after longer hypoxia durations (e.g. 48 h of 0.1% O_2) [39].

484 Melatonin induces BeWo cell death, in contrast to the effects of autophagy activation by
485 rapamycin. This could suggest that melatonin inhibits autophagy in BeWo cells, which is
486 supported by the present results showing decreased SQSTM1/P62 protein level in BeWo cells
487 treated with melatonin or BAF A1. Coto-Montes et al. [40] proposed that melatonin inhibits
488 autophagy via direct inhibition of upstream factors, such as the inflammatory factor, c-Jun N-
489 terminal kinase (JNK). Consequent to JNK inhibition, autophagy activation has been shown to
490 be decreased in a hepatoma cell line [41]. Other studies indicate that the inhibitory effect of
491 melatonin on autophagy may be mediated by the activation of endoplasmic reticulum (ER)-stress
492 [42, 43]. The lack of variation in the levels of LC3B lipidation in melatonin-treated BeWo cells
493 is not related to the lack of autophagic activity, but rather arises as a consequence of the active
494 destruction of defective mitochondria via mitophagy [39]. We observe a reduction in
495 mitochondrial content in melatonin treated BeWo cells exposed to H/R, which may indicate
496 heightened levels of mitophagy, and which has been shown to be harmful for cancer cells [44,
497 45]. Our result showing the decreased mitochondrial content of cancer cells exposed to H/R and
498 treated with melatonin corroborates other studies that have found antiproliferative and pro-
499 apoptotic activity of melatonin, evident in both *in vitro* and *in vivo* models as well as in clinical
500 assays for an array of diverse cancers [19, 42, 43].

501 In both normal and cancer cells, Nrf2 activation induces the expression of antioxidant

enzymes [6]. In H/R conditions, melatonin reduces the nuclear content of Nrf2 in BeWo cells, thereby lowering the cells levels of protective antioxidants and contributing to increased cleavage of PARP and cell death. In contrast, vCTBs exposed to H/R and treated with melatonin showed increased nuclear translocation of Nrf2. As such, the differential effects of melatonin in normal and tumoral trophoblasts is associated with parallel changes in Nrf2 and autophagy, with both being decreased in H/R-exposed BeWo cells, but increased in H/R-exposed vCTB. The dual role of melatonin in such important mechanisms of cell defense – Nrf2, and autophagy – indicates a co-ordinated set of changes that is harmful in tumoral cells and protective in normal cells. The differential effects of melatonin in tumoral vs normal cells have been shown over a number of decades, leading to great interest in the anticancerous utility of melatonin, especially given its low toxicity, small size, and amphiphilic profile [17, 18]. However, the mechanisms underlying the differential effects of melatonin in normal vs cancer cells is still poorly understood. In breast cancer cells, melatonin alters the patterned expression of microRNAs and as well as DNA methylation. It requires investigation as to whether melatonin has significant differential impacts on such epigenetic processes in normal vs tumoral cells, including placental cells [35, 46]. Recent work by Hardeland highlights the importance of melatonin acts as an epigenetic modulator in different cell types [35], although this has still to be investigated in placental cells (**Figure 7**). In a recent review by Reiter et al. [19], the cytotoxicity of melatonin in cancer cells was described as possibly via receptor-dependent and receptor-independent actions, with both mechanisms leading to decreased cancer cells viability by inhibiting protective pathways that would be activated in suboptimal conditions, such as hypoxia and low levels of amino acids.

Figure 7: Melatonin acts differentially in tumoral and normal placental cells. (a) In the BeWo choriocarcinoma cell line, melatonin increases the phosphorylation of 5 'adenosine

monophosphate-activated protein kinase (AMPK), due to the reduction of ATP levels [50]. Melatonin inhibits autophagic activity as well as the activation of the nuclear factor (erythroid-derived 2) -like 2 (Nrf2) transcription factor, contributing to BeWo cell death. (b) In primary cell cultures of human placental villous cytotrophobasts (vCTBs), H/R disrupts the cellular homeostasis. Melatonin treatment induces activation of two pro-survival mechanisms: autophagy and Nrf2, which help to restore homeostasis and cell survival.

This study makes an important contribution to the understanding of the biological underpinnings that result in the differential effects of melatonin in tumoral vs normal placental cells. The data indicate that the differential effects of melatonin on Nrf2 and autophagy are intimately linked to this. Future research should investigate as to whether the changes in Nrf2 and autophagy are linked to epigenetic processes (such as microRNAs), mitochondrial factors (such as oxidative phosphorylation vs glycolysis) and alterations in endogenous melatonergic pathway regulation. In conclusion, the data show that the differential effects of melatonin on Nrf2 and autophagy are important to its protection of normal cells and increased apoptosis of tumor cells, including under H/R conditions.

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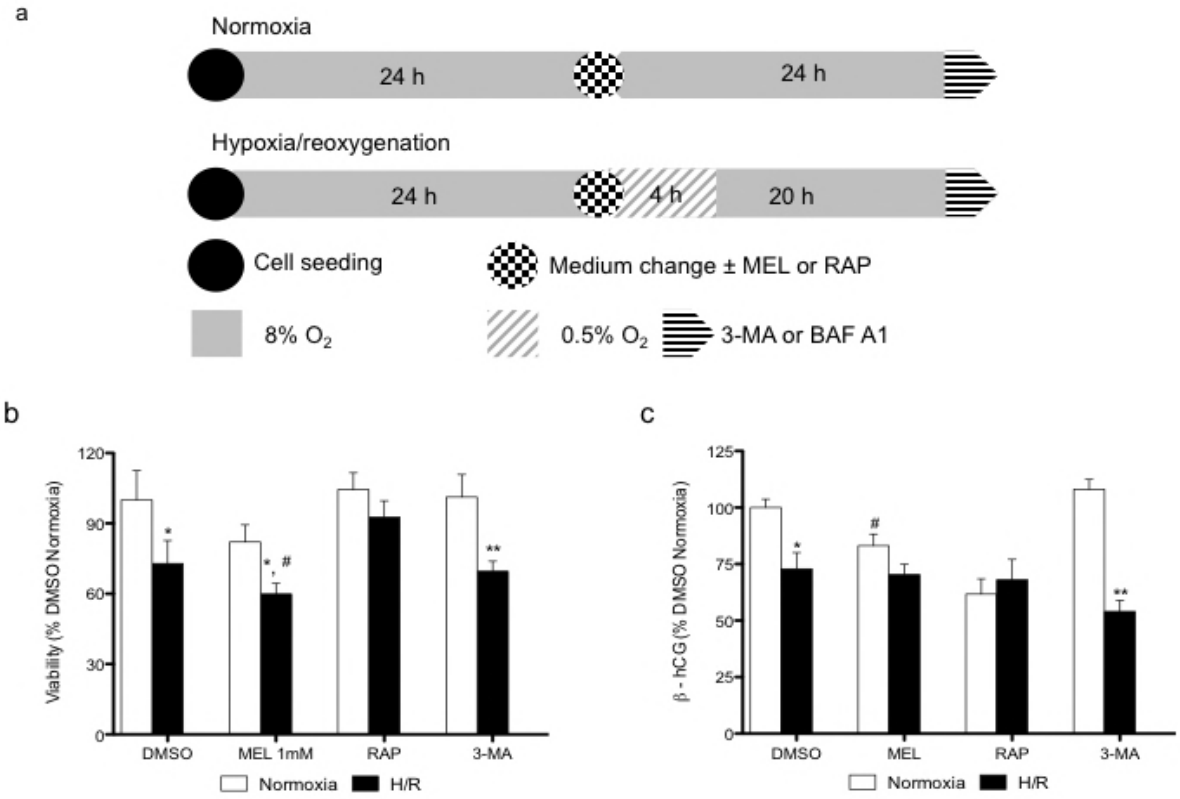
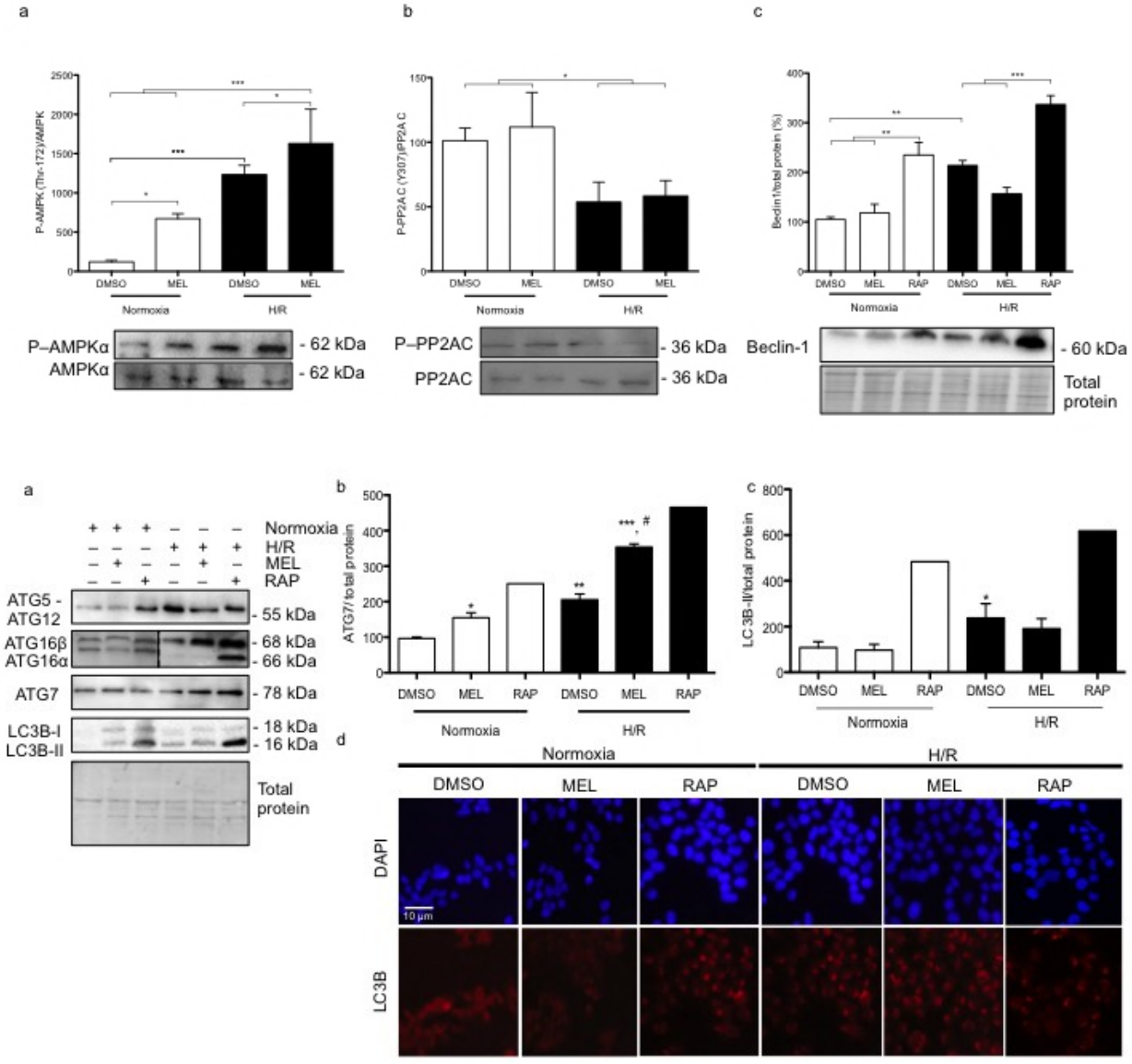
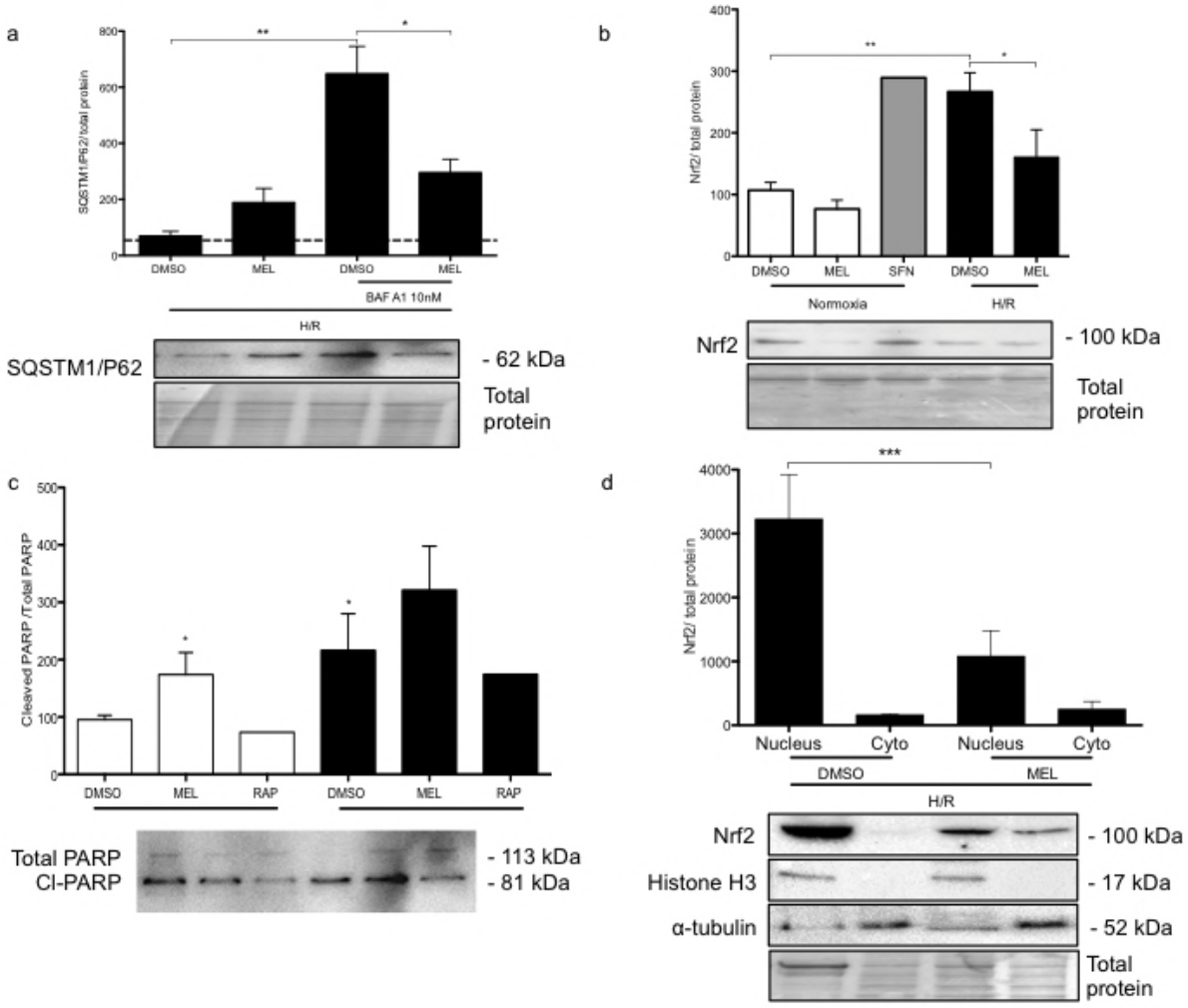
Supporting information

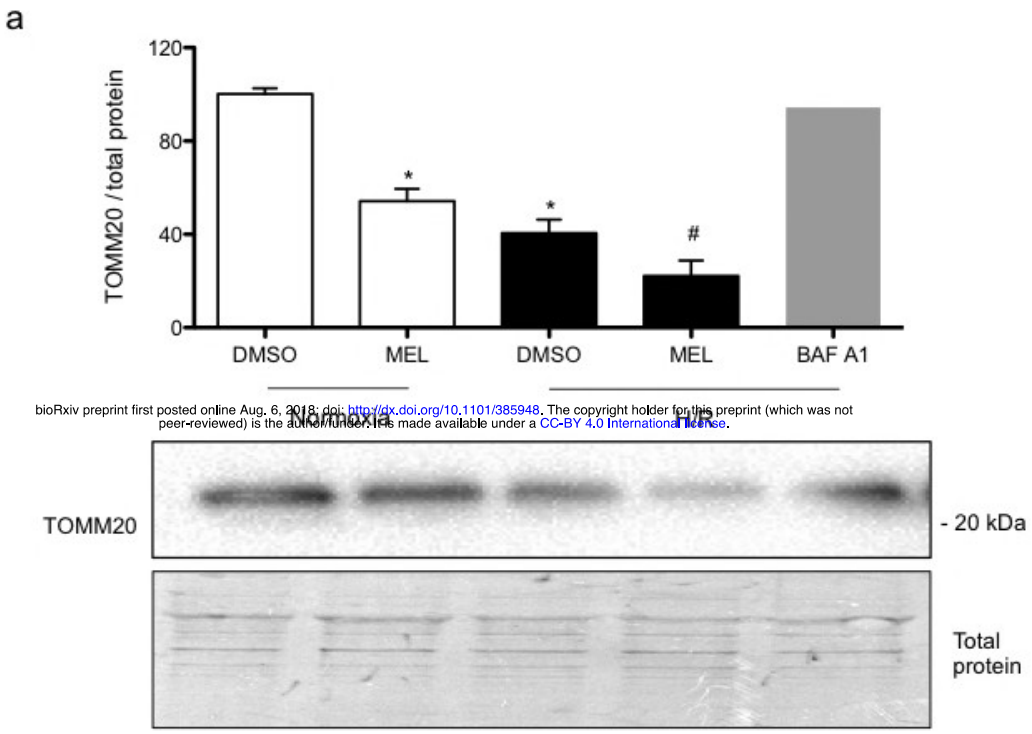
S1 Table. Antibodies used for Western blot analyses

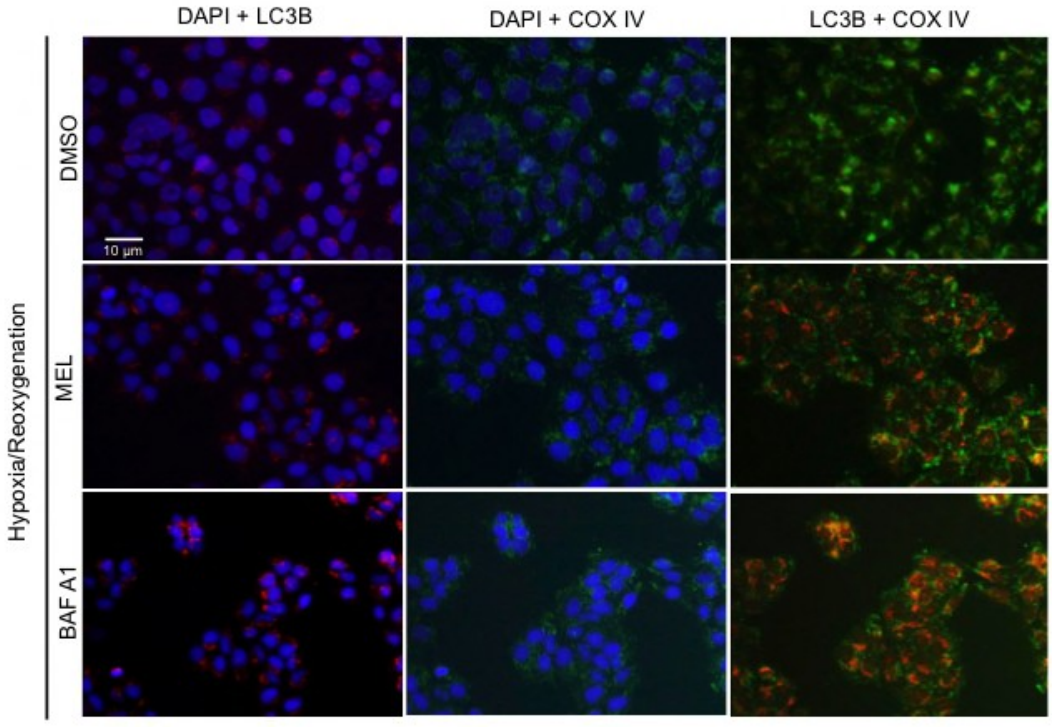
S2: Raw data of all of the results presented in this manuscript.

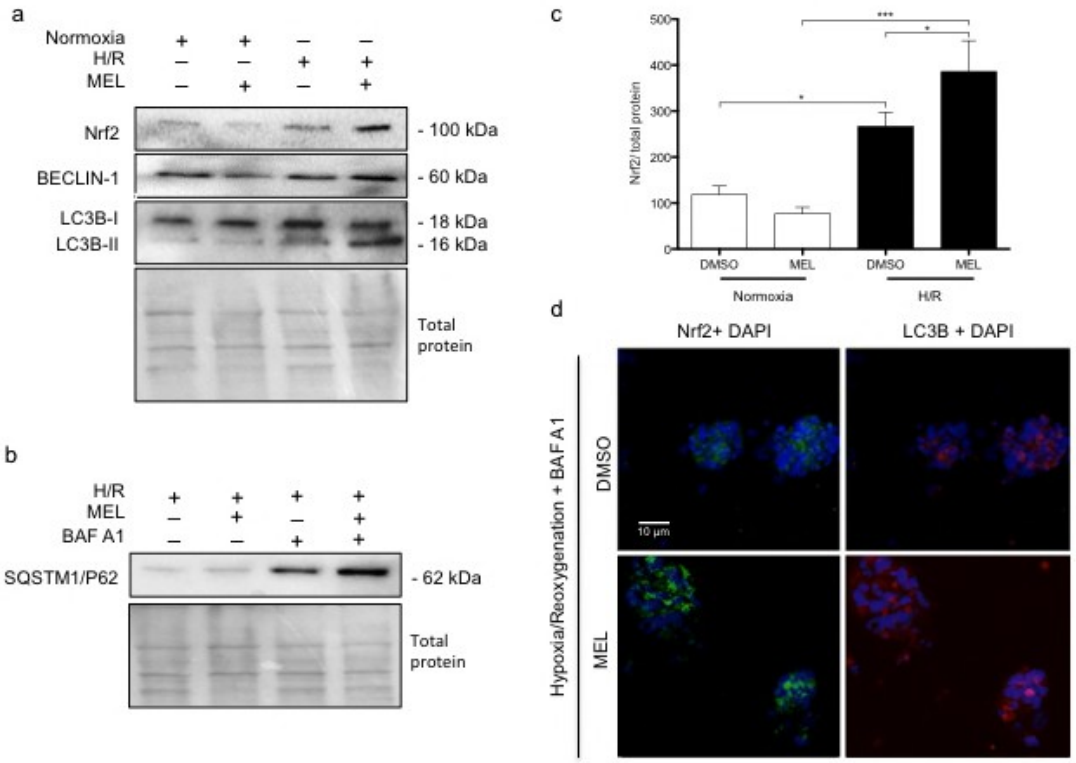


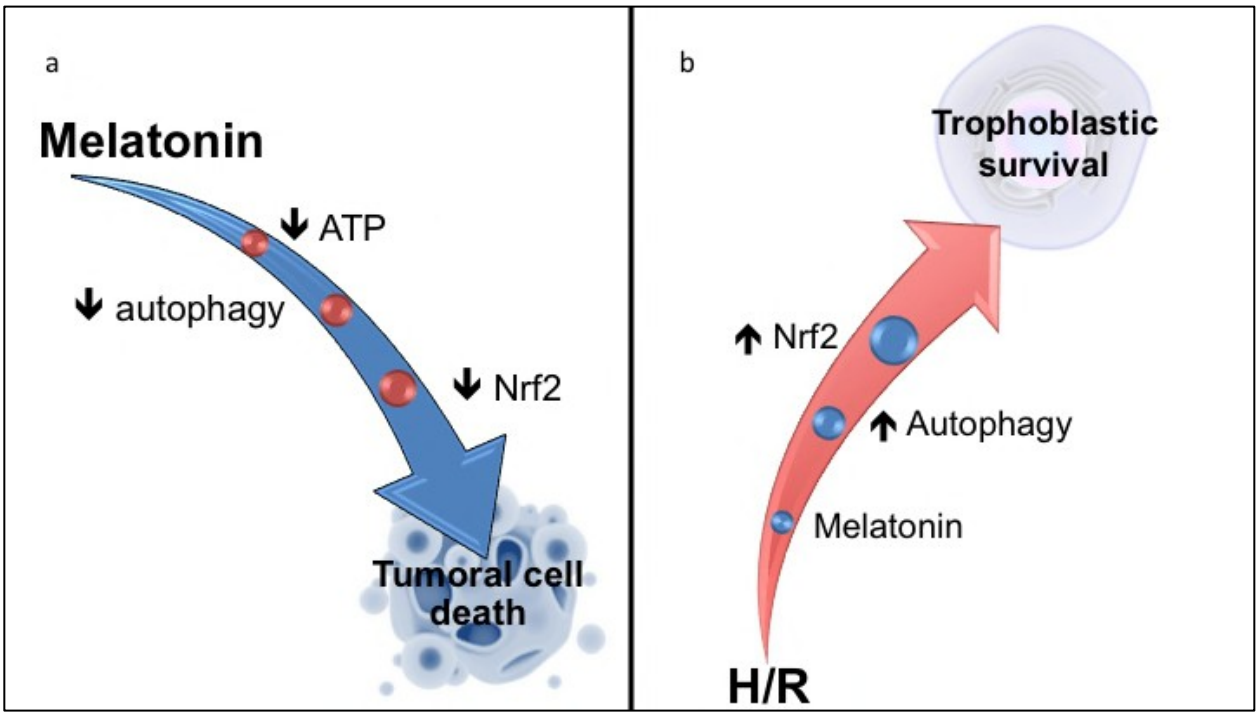

a
b
c
P-AMPK (Thr-172)/AMPK
P-PP2A C (Y307)/PP2A C
Beclin1/total protein (%)
DMSO
MEL
RAP
Normoxia
H/R
P–AMPKα
AMPKα
- 62 kDa
- 62 kDa
P–PP2AC
PP2AC
- 36 kDa
- 36 kDa
Beclin-1
- 60 kDa
Total protein
a
b
c
d
Normoxia
H/R
MEL
RAP
ATG5 -
ATG12
- 55 kDa
ATG16β
ATG16α
- 68 kDa
- 66 kDa
ATG7
- 78 kDa
LC3B-I
LC3B-II
- 18 kDa
- 16 kDa
Total protein
ATG7/ total protein
LC3B-II/total protein
DAPI
LC3B
10 μm


a
SQSTM1/P62/ total protein
DMSO
MEL
DMSO
MEL
BAF A1 10nM
H/R
SQSTM1/P62
- 62 kDa
Total protein
b
Nrf2/ total protein
DMSO
MEL
SFN
DMSO
MEL
Normoxia
H/R
Nrf2
- 100 kDa
Total protein
c
Cleaved PARP /Total PARP
DMSO
MEL
RAP
DMSO
MEL
RAP
Total PARP
Cl-PARP
- 113 kDa
- 81 kDa
d
Nrf2/ total protein
Nucleus
Cyto
Nucleus
Cyto
DMSO
MEL
H/R
Nrf2
- 100 kDa
Histone H3
- 17 kDa
α-tubulin
- 52 kDa
Total protein









Manuscript entitled: Melatonin: the smart molecule that differentially modulates autophagy in tumor and normal placental cells

Raw data files and statistical analysis used for generation of Figs 1 – 6

BeWo viability measurement (Fig. 1b)

	Normoxia			H/R		
Group	Average	Std dev	N	Average	Std dev	N
DMSO	100.000000	12.500000	9	72.873270	9.766103	9
MEL 1mM	82.051930	7.320060	9	59.864520	4.590479	9
RAP	104.449600	7.148348	9	92.661190	6.934567	7
3-MA	101.248900	9.644550	9	69.563930	4.319417	7

Parameter				
Table Analyzed	Data 1			
One-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	4.47	0.0072		
Column factor	43.99	< 0.0001		
Row factor	31.28	< 0.0001		
Source of Variation	P value summary	Significant ?		
Interaction	**	Yes		
Column factor	***	Yes		
Row factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	3	918.4	306.1	4.411
Column factor	1	9040	9040	130.3
Row factor	3	6429	2143	30.88
Residual	60	4164	69.39	
Student's *t*-test				
Normoxia vs H/R				
Row factor	Normoxia	H/R	Difference	95% CI of diff.
DM	100.0	72.87	-27.13	-37.24 to -17.01
MEL 1	82.05	59.86	-22.19	-32.30 to -12.07
R	104.4	92.66	-11.79	-22.60 to -0.9774
3-	101.2	69.56	-31.68	-42.50 to -20.87
Row factor	Difference	t	P value	Summary
DMSO	-27.13	6.908	P<0.01	**
MEL 1mM	-22.19	5.650	P<0.01	**
RAP	-11.79	2.808	P < 0.05	#, *

3-MA	-31.68	7.547	P<0.01	**

BeWo HCG secretion (Fig. 1c)

	Normoxia			H/R		
Group	Average	Std dev	N	Average	Std dev	N
DMSO	100.000000	8.281730	5	72.873270	14.272600	4
MEL	83.132580	11.435730	5	70.363710	9.230582	4
RAP	61.672200	15.093210	5	68.064300	18.019990	4
3-MA	108.070900	9.920430	5	53.955200	9.936420	4

Table Analyzed	Data 1			
One way-ANOVA				
Source of Variation	% of total variation	P value		
Interaction	28.73	0.0002		
Column factor	28.29	< 0.0001		
Row factor	14.86	0.0071		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column factor	***	Yes		
Row factor	**	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	3	4331	1444	9.535
Column factor	1	4265	4265	28.17
Row factor	3	2241	747.0	4.934
Residual	28	4239	151.4	
Student's *t*-test				
Normoxia vs H/R				
Row factor	Normoxia	H/R	Difference	95% CI of diff.
DMSO	100.0	72.87	-27.13	-49.16 to -5.092
MEL	83.13	70.36	-12.77	-34.80 to 9.266
RAP	61.67	68.06	6.392	-15.64 to 28.43
3-MA	108.1	53.96	-54.12	-76.15 to -32.08
Row factor	Difference	t	P value	Summary
DMSO	-27.13	3.286	P < 0.05	*

MEL	-12.77	1.547	P > 0.05	ns
RAP	6.392	0.7744	P > 0.05	ns
3-MA	-54.12	6.556	P<0.01	**

BeWo expression of Phospho-AMPK/Total AMPK (Fig. 2A)

Normoxia		H/R	
DMSO	MEL	DMSO	MEL
161.00000	768.24690	1419.707	768.069
100.00000	553.02540	1020.191	1942.941
100.00000	693.02770	1262.880	2178.310
115.00000	684.39550	1080.15620	1614.94150

One-way analysis of variance				
P value	0.0070			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	8.577			
R squared	0.7628			
ANOVA Table	SS	df	MS	
Treatment (between columns)	4009000	3	1336000	
Residual (within columns)	1246000	8	155800	
Total	5255000	11		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P < 0.05?	Summary
DMSO vs MEL	-1530	6.713	Yes	***
DMSO vs DMSO	-1134	4.977	Yes	***
DMSO vs MEL	-571.4	2.508	Yes	*
MEL vs MEL	-958.3	4.205	Yes	***

BeWo expression of Phospho-PP2Ac/Total PP2Ac (Fig. 2B)

Normoxia		H/R	
DMSO	MEL	DMSO	MEL
100.00000	184.39550	69.24640	66.1707
100.00000	84.90611	88.47250	83.3254
100.00000	116.69590	19.85130	57.7634
100.00000	60.89893	36.94590	26.0109

Table Analyzed	Data 1			
One-way analysis of variance				
P value	0.0349			
P value summary	*			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	2.670			
R squared	0.4003			
ANOVA Table	SS	df	MS	
Treatment (between columns)	133800	3	44610	
Residual (within columns)	200500	12	16710	
Total	334300	15		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
MEL vs MEL	-241.6	6.713	Yes	*
DMSO vs DMSO	-261.9	4.977	Yes	*
MEL vs DMSO	-2.026	2.508	No	ns
DMSO vs MEL	-199.6	4.205	No	ns

BeWo expression of Beclin-1 (Fig. 2C)

Normoxia			H/R		
DMSO	MEL	RAP	DMSO	MEL	RAP
100.	72.19473	185.4052	235.09070	196.74650	372.19470
100.	137.27550	265.5019	187.45670	177.75310	325.40520
100.	155.64900	254.8342	211.75390	133.75220	312.53290
100.	77.62914		220.76430	138.85370	
100.	147.44380			136.98770	

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	30.49				
R squared	0.8892				
ANOVA Table	SS	df	MS		
Treatment (between columns)	137400	5	27470		
Residual (within columns)	17120	19	901.0		
Total	154500	24			
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
DMSO vs MEL	-13.04	0.9713	No	ns	-73.03 to 46.95
DMSO vs DMSO	-108.8	7.639	Yes	***	-172.4 to -45.14
DMSO vs MEL	-51.82	3.860	No	ns	-111.8 to 8.173
MEL vs DMSO	-95.73	6.723	Yes	**	-159.4 to -32.10
MEL vs MEL	-38.78	2.889	No	ns	-98.77 to 21.21
MEL vs RAP	-218.7	14.11	Yes	***	-287.9 to -149.4
RAP vs DMSO	21.48	1.325	No	ns	-50.97 to 93.93
RAP vs MEL	78.43	5.060	Yes	***	9.156 to 147.7
RAP vs RAP	-101.5	5.855	Yes	**	-178.9 to -24.02
DMSO vs MEL	56.95	4.000	No	ns	-6.683 to 120.6
DMSO vs RAP	-122.9	7.584	Yes	***	-195.4 to -50.50
MEL vs RAP	-179.9	11.61	Yes	***	-249.2 to -110.6

BeWo protein content of ATG7 (Fig. 3B)

Normoxia			H/R		
DMSO	MEL	RAP	DMSO	MEL	RAP
100.	177.87890	250.4597	237.4819	362.00850	465.833800
100.	103.32250		204.5394	343.50370	
100.	203.35260		151.0475	320.78190	
100.	145.46940		233.0573	346.86170	
100.	144.46480		242.9475	376.31620	
100.	155.38240		165.8373	374.46480	

One-way analysis of variance				
P value	< 0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
P value	90.21			
One-way analysis of variance	0.9312			
P value	0.0410			
P value summary	*			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	218500	3	72840	
Residual (within columns)	16150	20	807.5	
Total	234700	23		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
DMSO vs MEL	-257.3	22.18	Yes	***
DMSO vs DMSO	-109.2	9.410	Yes	***
DMSO vs MEL	-58.32	5.028	Yes	**
MEL vs MEL	-199.0	17.16	Yes	***
MEL vs DMSO	-50.84	4.383	Yes	**
DMSO vs MEL	-148.2	12.77	Yes	***

BeWo LC3B-II protein content (Figure 3c)

Normoxia			H/R		
DMSO	MEL	RAP	DMSO	MEL	RAP
100.00000	11.93300	483.582200	242.323300	386.37500	617.85540
100.00000	137.09950		147.148900	196.13620	
100.00000	64.82899		208.212600	156.27960	
106.60000	190.70550		305.027700	212.20170	
23.56928	90.39797		486.107600	96.39732	
218.92110	87.92138		35.003300	98.99800	

One-way analysis of variance				
P value	0.042074			
P value summary	*			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	1.573			
R squared	0.2548			
ANOVA Table	SS	df	MS	
Treatment (between columns)	93230	5	18650	
Residual (within columns)	272700	23	11860	
Total	366000	28		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
DMSO N vs MEL H/R	-123.4	---	No	ns
DMSO N vs DMSO H/R	-151.7	6.726273	No	ns
DMSO N vs MEL N	-80.96	---	No	ns
MEL N vs MEL H/R	-42.48	---	No	ns
MEL N vs DMSO H/R	-30.77	---	No	ns

BeWo Protein expression of P62 in H/R conditions (Fig 4a)

H/R			
No BAF A1		BAF A1 10nM	
DMSO	MEL	DMSO	MEL
77.7253	111.7384	726.51350	306.7260
86.1010	126.7861	603.56410	254.3837
113.2395	114.9460	402.16380	422.3635
19.8947	225.1472	860.99500	201.1928
55.9840	369.4043		

Table Analyzed	Data 1			
One-way analysis of variance				
P value	< 0.001			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	19.25			
R squared	0.8049			
ANOVA Table	SS	df	MS	
Treatment (between columns)	803700	3	267900	
Residual (within columns)	194800	14	13920	
Total	998500	17		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
DMSO vs DMSO	-577.7	10.32	Yes	**
DMSO vs MEL	-225.6	4.031	Yes	*
DMSO vs MEL	-119.0	2.256	No	ns
MEL vs MEL	-106.6	1.904	No	ns

BeWo Protein expression of Nrf2 (Fig 4b)

Normoxia			H/R	
DMSO	MEL	SFN 10 µM	DMSO	MEL
150.00000	42.83741	116.8235	314.3438	269.65500
85.00000	65.88973	242.7504	242.7504	107.25200
120.00000	89.85131	445.6733	306.0482	193.85930
100.00000	108.53870	352.3331	156.3648	72.23429
80.00000			314.4300	

One-way analysis of variance				
P value	0.0057			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	5			
F	5.345			
R squared	0.5571			
ANOVA Table	SS	df	MS	
Treatment (between columns)	154300	4	38580	
Residual (within columns)	122700	17	7217	
Total	277000	21		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
MEL vs DMSO	-190.0	4.715	Yes	*
MEL vs MEL	-109.0	2.565	No	ns
MEL vs DMSO	-30.22	---	No	ns
DMSO vs DMSO	-159.8	4.206	Yes	*
DMSO vs MEL	-78.75	---	No	ns
MEL vs DMSO	-81.04	---	No	ns

BeWo Protein expression of Cleaved PARP-1/PARP-1 (Fig 4c)

Normoxia			H/R		
DMSO	MEL	RAP	DMSO	MEL	RAP
100.000000	91.41441	62.801240	66.87990	180.64420	174.83400
100.000000	199.50970	211.514600	338.53260	120.19050	95.45602
100.000000	300.94960	3.490018	287.26050	519.35900	287.01450
100.000000	181.51460	62.801240	332.97430	453.09430	195.06500
100.000000	95.90380	25.812400	55.98500	333.95300	120.08500

Table Analyzed	Data 1			
One-way analysis of variance				
P value	0.0367			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	4			
F	3.094			
R squared	0.3671			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	12.62			
P value	0.0055			
P value summary	**			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	133100	3	44380	
Residual (within columns)	229500	16	14340	
Total	362700	19		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
DMSO vs MEL	-226.0	4.220	Yes	*
DMSO vs DMSO	-220.9	4.258	Yes	*
DMSO vs MEL	-278.46	5.736	Yes	*
MEL vs MEL	-147.6	2.755	No	ns
MEL vs DMSO	-42.47	---	No	ns
DMSO vs MEL	-105.1	---	No	ns

BeWo Protein expression of Nrf2 (Nuclear and cytosolic fractions) (Fig 4d)

H/R			
DMSO		MEL	
Nucleus	Cyto	Nucleus	Cyto
2986.68400	133.06750	2226.4710	123.68240
2293.20000	171.38590	400.7336	113.98630
5263.56000	188.23100	700.9383	615.38370
2373.94800	154.83830	1000.8370	145.83730

Table Analyzed	Data 1
Column Nucleus DMSO	Nucleus
vs	vs
Column Nucleus Melatonin	Nucleus
Unpaired t test	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.675 df=6
How big is the difference?	
Mean ± SEM of column A	3229 ± 395.5 N=4
Mean ± SEM of column C	1082 ± 400.6 N=4
Difference between means	2147 ± 802.6
95% confidence interval	183.0 to 4111
R squared	0.5439
F test to compare variances	
F,DFn, Dfd	3.014, 3, 3
P value	0.3890
P value summary	ns
Are variances significantly different?	No

BeWo Protein expression of TOM20 (Fig. 5a)

Normoxia		H/R		
DMSO	MEL	DMSO	MEL	BAF A1
100.	59.93741	26.17530	4.43680	94.40770
100.	76.30316	57.49330	40.46980	
100.	46.39102	38.05123	21.72150	
100.	39.93741	26.17530	4.43680	
100.	46.30316	57.49330	40.46980	
100.	56.39102	38.05123	21.72150	

Parameter				
Table Analyzed	Data 1			
One-way analysis of variance				
P value	< 0.0263			
P value summary	*			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	41.96			
R squared	0.8629			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)				
P value				
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	19840	3	6613	
Residual (within columns)	3152	20	157.6	
Total	22990	23		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
MEL vs DMSO	-77.79	15.18	Yes	*
MEL vs MEL	-32.00	6.244	Yes	*
MEL vs DMSO	-18.36	3.583	Yes	*
DMSO vs DMSO	-59.43	11.60	Yes	*
DMSO vs MEL	-13.64	2.661	No	ns
MEL vs DMSO	-45.79	8.934	Yes	*

Human primary villous cytotrophoblast Nrf2 expression (Fig. 6b)

Normoxia		H/R	
DMSO	MEL	DMSO	MEL
100.00000	42.83741	314.3438	269.65500
100.00000	65.88973	242.7504	307.25200
100.00000	89.85131	306.0482	393.85930
100.00000	108.53870	156.3648	572.23430
		314.4300	

Parameter				
Table Analyzed	**Data 1**			
One-way analysis of variance				
P value	0.0002			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	14.81			
R squared	0.7737			
ANOVA Table	**SS**	**df**	**MS**	
Treatment (between columns)	259100	3	86350	
Residual (within columns)	75780	13	5829	
Total	334800	16		
Newman-Keuls Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
MEL vs MEL	-309.0	8.094	Yes	***
MEL vs DMSO	-190.0	5.247	Yes	**
MEL vs DMSO	-23.22	0.6083	No	ns
DMSO vs MEL	-285.8	7.486	Yes	***
DMSO vs DMSO	-166.8	4.606	Yes	**
DMSO vs MEL	-119.0	3.285	Yes	*

	DMSO	**MEL**	**DMSO**	**MEL**
Number of values	**4**	**4**	**5**	**4**
Minimum	100.0	42.84	156.4	269.7
25% Percentile	100.0	48.60	199.6	279.1
Median	100.0	77.87	306.0	350.6
75% Percentile	100.0	103.9	314.4	527.6
Maximum	100.0	108.5	314.4	572.2
Mean	100.0	76.78	266.8	385.8
Std. Deviation	0.0	28.58	68.64	134.8
Std. Error	0.0	14.29	30.70	67.38
Lower 95% CI	100.0	31.30	181.6	171.3
Upper 95% CI	100.0	122.3	352.0	600.2

5. RÉSULTATS PRÉLIMINAIRES: CD95 REGULATES THE HUMAN PLACENTAL HOMEOSTASIS

Toutes les analyses présentées dans le chapitre 5 ont été réalisées dans le laboratoire dirigé par le Dr Patrick Legembre (INSERM U1242 (Laboratoire d'Oncogenèse, Stress et Signalisation), Université Rennes 1, France). Son laboratoire se concentre sur les aspects moléculaires du système CD95 (également connu sous le nom de Fas), impliqué dans la modulation de l'auto-immunité et de l'oncologie. La tolérance maternelle par rapport au placenta est contrôlée par le système immunitaire, y compris le système CD95.

Contribution de l'étudiant

L'étudiant a écrit les deux demandes de bourses de stage doctorale, ERASMUS + et RQR-FRQNT, qui ont permis la réalisation du stage. Le sujet de stage est l'idée de l'étudiant, projet qu'il a proposé aux chercheurs impliqués (Dre Vaillancourt (INRS-IAF) et Dr Legembre (UR1)). L'étudiant a réalisé la totalité des expériences et analysé tous les résultats. Il a également rédigé le chapitre 5.

Résumé en français

L'activation de CD95 (Fas) via son ligand natif, le CD95L (Fas ligand), peut déclencher une inflammation par activation des voies NFkB et MAPK ou apoptose via le clivage de la caspase 8. Le CD95 est un régulateur clé de la réponse immunitaire. En effet, l'inactivation ou la déplétion de ce récepteur est positivement associée à la sévérité de l'auto-immunité. Le CD95L est exprimé dans le placenta et joue un rôle important dans le privilège immunitaire de cet organe, probablement par l'induction de l'apoptose des lymphocytes maternels. Les objectifs de ce stage étaient de mieux comprendre le rôle de l'activation de CD95 / CD95L dans les explants de villosités isolés de placentas de premier trimestre de grossesse et à terme. En utilisant l'immunohistochimie, les explants du premier trimestre démontrent des niveaux plus élevés de CD95L et une très forte corrélation positive avec le clivage de la caspase 3 par rapport aux explants placentaires à terme. L'expression du CD95 est stable au premier trimestre et à terme. L'H/R entraînent une augmentation des taux de CD95L, induisant l'apoptose via l'activation des caspases -8 et -3 dans les explants de placentas à terme par rapport à la normoxie. Sous H/R, le traitement à la mélatonine inhibe le clivage excessif du CD95L et de la caspase 3 et les ramène au niveau basal (c.-à.-d. en normoxie). Le système CD95 est activé de manière variable dans le placenta en ce qui concerne l'avancement de la grossesse et la variation d'oxygénation et la mélatonine apparemment est un puissant modulateur de du CD95L, avec des conséquences sur la modulation de l'apoptose extrinsèque via CD95.

MATERIAL AND METHODS

Placental explant cultures

First trimester and term placentas were donated by healthy women undergoing elective termination of pregnancy (9–14 weeks of gestation) or from normal term pregnancy (≥38 weeks) from women undergoing caesarean delivery. First trimester villous explants were collected from Hôpital Cochin (Paris, France) after ethical approval and informed consent (CCP- PRB, Paris Cochin, N°18–05, Paris, France). Term villous explants were collected from Centre Hospitalier Universitaire Sainte-Justine (CHUSJ, Montreal, Canada) after ethical approval and informed consent. Villous explants were prepared and cultured as previously described (Miller et al. 2005). Briefly, placental membranes were removed, then placental villi were washed three consecutive times in DMEM baths, followed by dissection in ~10 mg wet weight pieces of chorionic villi in Hank's Balanced Salt Solution. First trimester explants were suspended, using a needle, above wells filled with OptiMEM medium and cultured at 37 °C, 20 % O_2 and 5 % CO_2 for 72 h in a 24-wells plate. Term explants were placed on inserts in a 24-wells plate filled with OptiMEM medium and cultured at 37 °C, 20 % O_2 and 5 % CO_2 for 72 h. The sizes of the tissue pieces (2–3 mm) were similar within wells, between wells and also between placentas. Term explants were divided in two distinct groups regarding the oxygenation levels of culture. A set of explants was cultured in 20 % O_2 to match the levels of oxygenation in which the first trimester explants were maintained (as described above). Another group of term explants was exposed to normoxia (8 % O_2, 5 % CO_2, 87 % N_2) in Modular Incubator Chambers (Billups-Rothenberg, San Diego). Every 24 h, explants maintained in normoxia had the medium changed and were either treated with 1 mM melatonin (Sigma-Aldrich, Oakville, Canada) or vehicle control (Dimethyl sulfoxide (DMSO) 0.1 %). After 48 h of culture under normoxia cells were either exposed to normoxia for more 24 h or exposed to hypoxic conditions (0.5 % O_2, 5 % CO_2, 94.5 % N_2) during 4 h hours followed by 20 h of normoxia, creating a hypoxia/reoxygenation (Sagrillo-Fagundes et al. 2016). Time “zero” was the time of plating, then explants were kept in culture for 24 h. Medium was renewed every 24 h, and the collected media stored at −80°C for future analysis. After 72 h of culture, the explants were stored at −80°C for future analysis or were washed with 1% PBS, fixed with 4% paraformaldehyde, and embedded in paraffin.

Immunoblotting

Briefly, frozen villous explants were lysed with ice-cold radioimmunoprecipitation (RIPA)

buffer (50 mM Tris-HCl pH 7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) containing protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were extracted on ice with periodic vortexing every 5 min during 30 min and then sonicated. The homogenate was then centrifuged at 14 000 g for 10 min at 4 °C. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Waltham, MA). Proteins (30 - 50 µg protein) were separated on Mini-PROTEAN® TGX™ Precast Protein Gels (4–15% precast polyacrylamide gel ; Bio-Rad, Saint Laurent, Canada), followed by transfer into Polyvinylidene fluoride (PVDF) membranes (Bio-Rad), blocked with casein buffer (1 %) and then immunoblotting with the specific primary antibodies: cleaved caspase-3 (Cell signaling Technologies, Denvers, MA ; 1:500 overnight, 4 ^{o}C); CD95 (R&D systems, Minneapolis, MN; 1:1000 overnight, 4 ^{o}C); CD95L (Cell Signaling Technologies; 1:500 overnight, 4 ^{o}C); caspase-8 (Cell Signaling Technologies; 1:2000 overnight, 4 ^{o}C); and X-linked inhibitor of apoptosis protein (XIAP ; Cell Signaling; 1:2000 overnight, 4 ^{o}C). Membrane were then incubated with specific secondary antibodiescoupled with horseradish peroxidase (HRP). Blots were developed with enhanced chemiluminescence reagent (Bio-Rad). Expressed proteins were normalized with β-actin (Cell Signaling Technologies; 1:5000 overnight, 4 ^{o}C) and quantified using the ImageJ software (Bethesda, MA).

Immunohistochemistry

First trimester and term villous explants were fixed in 4 % paraformaldehyde for 12 h. The tissues were incubated for 1 h in each of the following solution: 80% ethanol (twice), 95% ethanol (twice), 100% ethanol (twice), xylene 100% (three times). Then, explants were incubated in a 1:1 xylene/paraffin solution overnight. The tissue was then embedded at 58°C four times in paraffin wax (Oxford Labware, St. Louis, MO) for 90 min to form tissue blocks. Four-micrometer sections were stained by the automated system DISCOVERY XT (Roche ®, Illkirch, France). The buffer CC1 ® (Tris pH 8) was used to obtain the antigenic retrieval and 0.03% H_2O_2 in PBS was used for extinguishing the endogenous peroxidase activity. Primary and secondary antibodies were used as described in the Table 1. After the incubation with specific antibodies, slides were included in diaminobenzydine (OmniMap DAB® Roche) and counterstained with Mayer's haematoxylin. High-resolution images were captured with Hamamatsu Nanozoomer 2.O RS. Quantitative analyses of CD95 ligand and cleaved caspase 3 were detected with the *NDP.analyse* software (Hamamatsu Photonics, Hamamatsu, Japan) and were based on the immunostained area over the tissue area. The thresholds of the total tissue

area and of the immunostained area were validated and then applied for every sample (Gillespie et al. 2002; Gevaert et al. 2015).

Statistical analysis

All data represent at least three different primary cell cultures. Statistically significant differences ($p \leq 0.05$) of parametric results were determined by one-way analysis of variance (ANOVA) and identified by the *post hoc* test of Student-Newman-Keuls. Non-parametric data were analyzed using Kruskal-Wallis test, followed by *post hoc* Dunn's test. Data were analyzed using GraphPad Prism (version 5.04).

Table 1 : Table listing the details of the antibodies used in the immunohistochemistry

Antibody	Manufacturer	Host	Cat. number	Dilution	Incubation
CD95L	BD Pharmingen	Mouse	556387	1/400	O/N 4°C
Cl-caspase 3	Cell Signaling Technologies	Rabbit	9661	1/300	1 h 37°C
CD31	Abcam	Rabbit	ab28364	1/100	1 h 37°C
CK7	DAKO	Mouse	M7018	1/100	32 min. 37°C
CD163	Thermo Fisher	Mouse	M0701	1/300	32 min. 37°C

CD95L: CD95 ligand (CD178); Cl-caspase 3: Cleaved caspase-3; CK7: Cytokeratin 7.

RÉSULTATS

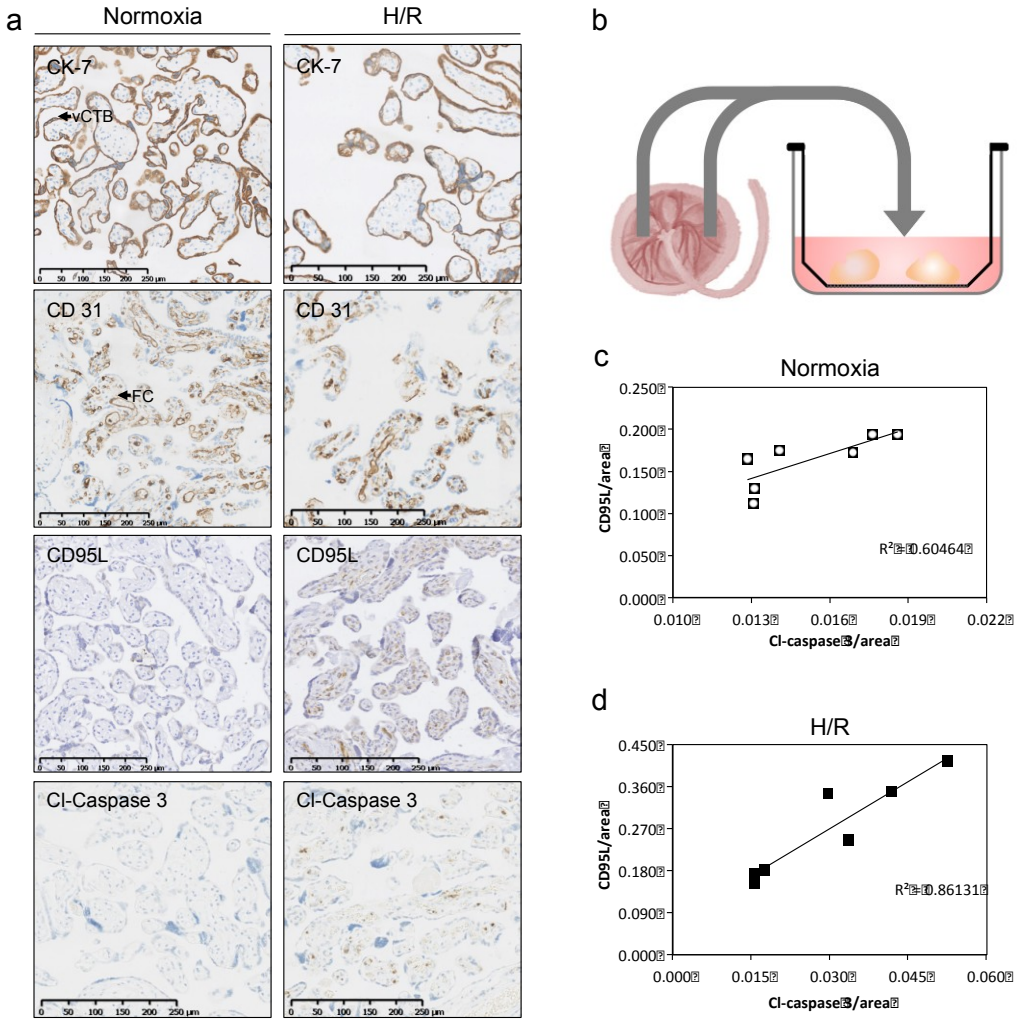


Figure 1. Hypoxia/Reoxygenation increases the levels of CD95 ligand and cleaved-caspase 3. Explants were obtained as described in material and methods. from normal term placentas (caesarean birth) and maintained for 72 h in normoxia (8% O_2; 5% CO_2; 87% N_2) or H/R (0.5% O_2; 5% CO_2; 94.5% N_2) protocols. (a) Left column shows placental explants immunodetection of cytokeratin–7 (CK–7), CD31, CD95 ligand (CD95L) and cleaved caspase 3. CK–7 was used to detect villous cytotrophoblasts (vCTB); CD31 was used to detect endothelial

cells (fetal capillaries (FC)). (b) Generic diagram of placental explants, kept in normoxia for 96 h, or 72 h in normoxia, followed by hypoxia (4 h) and reoxygenation (20 h) (H/R). (c - d) The regression graphs show the relative amount of CD95L compared to cleaved caspase-3 in normoxia (c) or H/R (d).

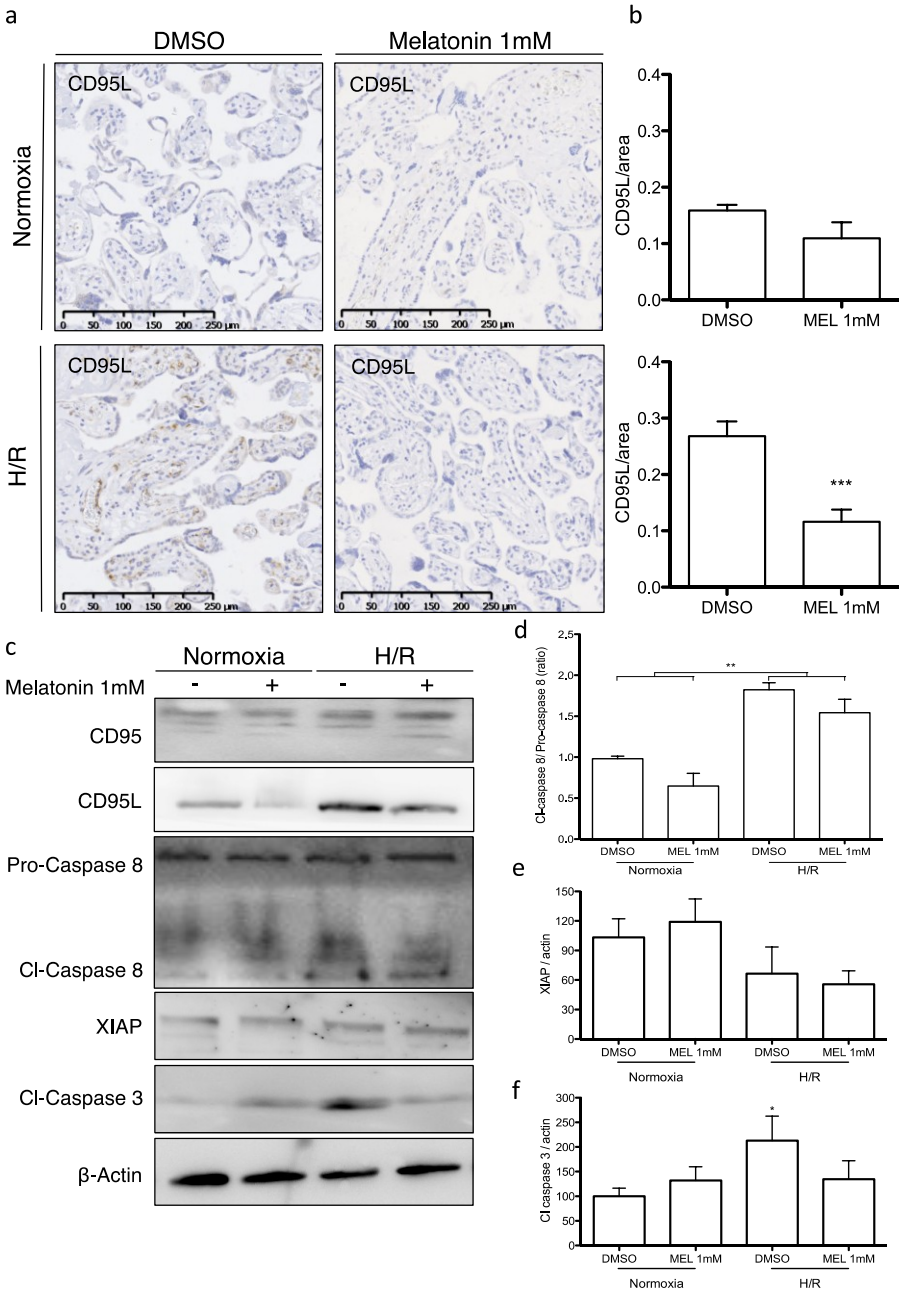


Figure 2. Melatonin prevents CD95 ligandand Caspase 3 cleavage upregulation in hypoxia/reoxygenation conditions. Explants were obtained from normal term placentas and maintained for 72 h in normoxia or H/R protocols. Explants media were changed every 24 h and

exposed to dimethyl sulfoxide (0.1 %) vehicle control or melatonin 1 mM. (a) Representative results of immunohistochemical analysis of CD95 ligand (CD95L) in term villous explants. (b) Quantitative analyses of CD95L immunodetection with the NDP.analyse software (Hamamatsu Photonics, Hamamatsu, Japan) were based on the immunostained area over the tissue area. Validated tissue and the immunostaining thresholds were previously validated and then applied for every sample. (c) CD95, CD95L, cleaved caspase 3, X-linked inhibitor of apoptosis protein (XIAP), and β-actin were detected by immunoblotting using a polyclonal specific antibody (table 1). Full-length and cleavage fragment of caspase 8 were detected with a monoclonal antibody. Bar graph represents the relative optical density of (d) cleaved caspase 8 normalized with full-length caspase 8 ; (e) XIAP normalized with β-actin ; (f) cleaved caspase 3 normalized with β-actin. Data shown are average ± SEM of five independent experiments and were analyzed using ANOVA, followed by Newman-Keuls. (* $P \leq 0.05$).

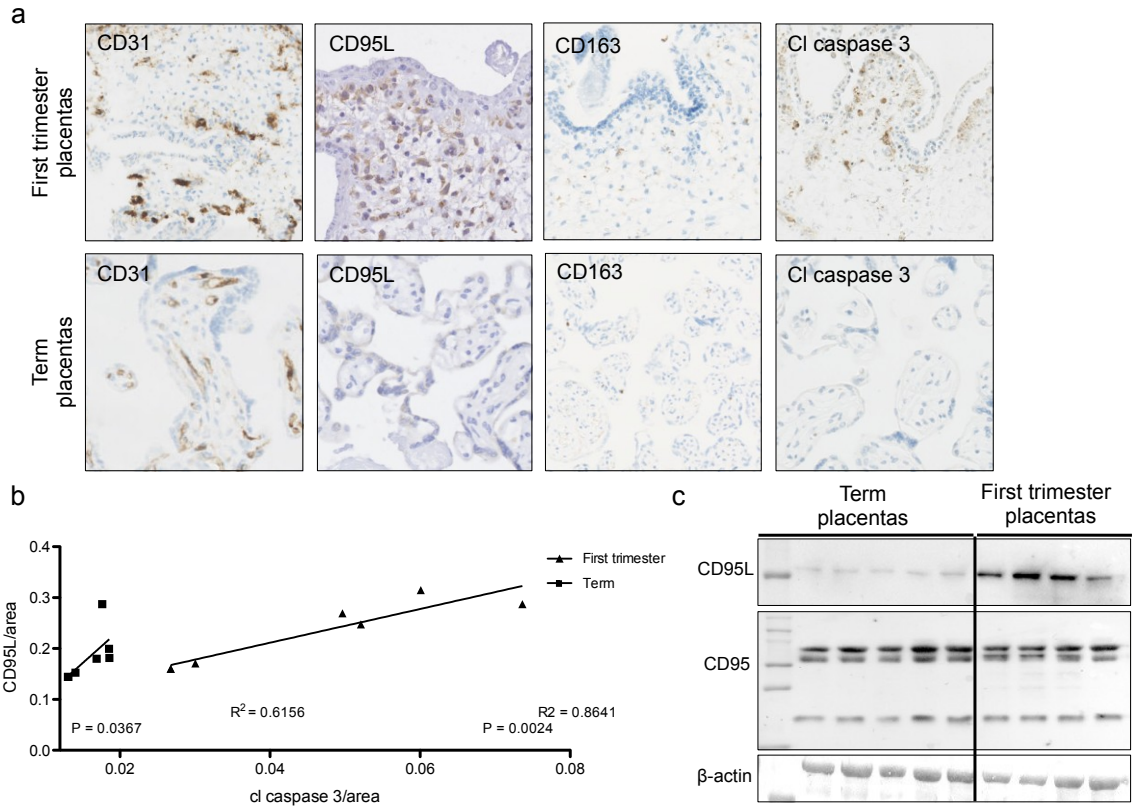


Figure 3. First trimester villous explants express higher levels of CD95 ligand compared to term explants. Explants were obtained from first trimester terminated pregnancies (8 – 13 weeks of pregnancy) or normal term placentas (38 – 41 weeks of pregnancy) and maintained for 72 h in culture. (a) Upper column shows placental explants immunodetection of cluster of differentiation 31 (CD31), CD95 ligand (CD95L), cluster of Differentiation 163 (CD163) and

cleaved caspase 3. CD31 was used to detect endothelial cells and CD163 is a phagocytic marker. (b) The regression graph shows the relative amount of CD95L compared to cleaved caspase-3 in first trimester and term explants. (c) CD95L, CD95, and β-actin were detected by immunoblotting using a polyclonal specific antibodies (Table 1).

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6. DISCUSSION ET CONCLUSIONS GÉNÉRALES

Les résultats présentés dans cette thèse démontrent dans les primocultures de vCTB que : (1) la mélatonine exerce une action cytoprotectrice contre les effets néfastes de l'H/R sans modifier la différenciation trophoblastique (**chapitres 2 et 3**); (2) l'H/R active la voie inflammatoire, l'autophagie et réduit la synthèse de mélatonine (**chapitres 3 et 5**). Cette thèse démontre également que (3) la mélatonine inhibe l'autophagie et induit l'apoptose, et donc a un effet cytotoxique de dans les cellules de choriocarcinome placentaire (**chapitre 4**). Les résultats sont en accord avec les hypothèses proposées, car ils démontrent le pouvoir de protection de la mélatonine sur les primocultures de trophoblastes villeux par l'autophagie et l'inflammation et au contraire la mélatonine a un effet cytotoxique par la régulation de l'autophagie dans les cellules trophoblastiques tumorales.

6.1 Justification du choix des modèles expérimentaux

Les travaux de cette thèse sont basés sur deux modèles de cellules trophoblastiques humaines : une lignée cellulaire issu de choriocarcinome (trophoblaste tumorale) et les primocultures de trophoblastes villeux isolées de placenta de grossesse normale à terme.

Le **chapitre 2**, *"Human Primary Trophoblast Cell Culture Model to Study the Protective Effects of Melatonin Against Hypoxia/reoxygenation-induced Disruption"*, présente le modèle de primoculture de trophoblaste villeux cultivé en condition normoxique (physiologique) et sous H/R (pathologique). Fondées sur le fait que le sang maternel baigne le STB dans l'espace intervilleux placentaire au cours du deuxième et du troisième trimestre de la grossesse, les cellules ont été exposées à des conditions optimales et sous-optimales d'oxygénation. Les conditions optimales d'oxygénation sont caractérisées par des taux constants de 60 mmHg qui *in vitro*, représente une atmosphère d'~8% O_2. Les conditions sous-optimales d'oxygénation sont une caractéristique des pathologies de grossesse associées avec des altérations placentaires, telles que la prééclampsie ou la restriction de croissance intra-utérine. Dans ces deux cas, les taux d'oxygénation varient de façon intermittente entre la normoxie (~ 8% O_2) et des taux extrêmement bas de 5 mmHg, (~0,5% O_2), ce qui crée une H/R. Les vCTBs ont été cultivées pendant 72 h pour qu'elles se différencient en STB. Une fois différencié, le STB a été exposé à une H/R ou normoxie et traité ou non avec la mélatonine. Le traitement continu avec la mélatonine 1mM n'a pas modifié la différenciation des vCTBs en STB. Noter que les taux d'oxygénations utilisés dans cette thèse ont été reproduits avec succès *in vitro*, par plusieurs

groupes, y compris le nôtre (Chen *et al.* 2012; Lanoix *et al.* 2013). Notre groupe avait déjà publié ce modèle en mettant l'accent sur l'augmentation du stress oxydatif et l'activation de l'apoptose dans le STB en condition d'H/R (Lanoix *et al.* 2013). Dans la présente étude, la caractérisation du modèle a été poursuivie en étudiant le profil inflammatoire et la voie autophagique. Dans le **chapitre 3**, on a pu démontrer que l'H/R induit des effets pro-inflammatoires, qui activent l'autophagie et une diminution de la synthèse endogène de la mélatonine dans les STB.

Pour déterminer si les effets de la mélatonine sont différents dans les cellules tumorales comparées aux cellules normales, deux phénotypes de cellules trophoblastiques ont été analysés : les primocultures de vCTBs et la lignée de choriocarcinome placentaire humain, les cellules BeWo**.** Les cellules BeWo, comme les primocultures, produisent la hCG et, lorsqu'exposées à un activateur de la voie de l'AMPc, elles se fusionnent en cellules multinucléées et forme un syncytium. Les cellules BeWo sont donc considérées comme un bon modèle pour étudier la syncytialisation, car une fois différentiées, elles expriment certains marqueurs de différenciation associés à la syncytialisation des vCTBs (Orendi et al. 2010; Ji et al. 2013). Les cellules BeWo sont de phénotypes épithéliaux et dérivent d'un choriocarcinome placentaire métastatique associé à un fœtus mâle. De plus en plus d'études ont démontré des différences entre les primocultures de vCTB et les cellules BeWo, liées entre autres à leur profil épigénétique, transcriptomique et protéomique (Burleigh *et al*. 2007; Novakovic *et al*. 2011). Notre équipe avait déjà démontré que la mélatonine a des effets différents dans les cellules BeWo et les primocultures de trophoblastes villeux (Lanoix *et al*. 2012). Dans les cellules BeWo la mélatonine induit l'apoptose intrinsèque tandis qu'elle a un effet cytoprotecteur dans les cellules primaires cultivées à 20 % O_2. En s'appuyant sur ces données et la littérature, dans la présente étude, les cellules BeWo et les primocultures de vCTB (mises en culture pendant 24 h), ont été utilisées pour comparer les effets de la mélatonine sur la voie de l'autophagie dans les cellules trophoblastiques normales comparées aux cellules tumorales en condition de normoxie et sous H/R (**chapitre 4**). Ces résultats pourraient potentiellement être extrapolés à d'autres types cellulaires normaux et tumoraux (Orendi *et al*. 2011; Roohbakhsh *et al*. 2018).

Ce projet doctoral a également permis le développement d'un modèle *ex vivo* d'explants de villosités placentaires isolées de grossesses normales de 1er trimestre et à terme cultivés sous normoxie et H/R **(chapitre 5)**. Comme décrit dans le **chapitre 5**, après 48 h de culture en normoxie, les explants ont été exposés à une H/R ou maintenus en normoxie et exposés ou non à 1 mM de mélatonine. Les figures 1 et 2 **(chapitre 5)** montrent que sous H/R, l'apoptose extrinsèque est fortement activée, via l'activation du CD95 et le clivage de la caspase 3. Plus

spécifiquement, CD95 ligand (CD95L) est significativement augmenté en H/R et est corrélé positivement avec la caspase 3 clivée. Quand les explants sont traités avec 1mM de mélatonine, le déclenchement de l'apoptose via l'activation du récepteur CD95 est significativement réduit (Figure 2, **chapitre 5**).

Dans ce projet de doctorat, l'H/R a été utilisée comme « stress » pour perturber l'homéostasie cellulaire et déterminer si la mélatonine renverse les effets de l'H/R. D'autres méthodes pour générer un stress cellulaire, comme un faible taux de glucose ou d'acides aminés, auraient pu être utilisées. Le choix de l'H/R est fondé sur le fait que ce stress mime les altérations cellulaires et moléculaires observées dans les placentas de grossesse pathologique, tels que la prééclampsie et la restriction de la croissance intra-utérine (Lanoix *et al*. 2013; Chen *et al.* 2012).

6.2. Les effets de l'H/R sur l'homéostasie des primocultures de trophoblaste villeux : action protectrice de la mélatonine

Le premier objectif de cette étude était de déterminer si et par quelles voies de signalisation la mélatonine régule l'inflammation et l'autophagie dans les primocultures de vCTB et de STB cultivées en normoxie et H/R. En accord avec notre hypothèse, les résultats présentés au **chapitre 3** démontrent dans le STB que la mélatonine renverse les effets pro-inflammatoires de l'H/R. De même, la mélatonine a augmenté l'expression de protéines effectrices de l'autophagie et l'activité de l'autophagie, entraînant une augmentation significative de la survie cellulaire et le maintien des fonctions cellulaires, telles que la production de hCG. Ces résultats sont en accord avec les données antérieures de notre équipe qui démontraient dans le STB un effet protecteur de la mélatonine contre les effets apoptotiques et oxydants de l'H/R. L'H/R augmente significativement l'autophagie dans le STB comparé à la normoxie. Cependant, cette augmentation de l'autophagie n'est pas suffisante pour contrecarrer l'augmentation de la mort du STB par apoptose. Tel que nous l'avions postulé, l'exposition du STB à 1 mM de mélatonine exogène sous H/R augmente l'activité autophagique, ce qui diminue la mort par apoptose au taux observé en normoxie.

Nos résultats montrent également que l'H/R augmente les taux de TNF et d'IL-6 sécrétés par le STB. Le TNF est le membre le plus étudié des cytokines pro-inflammatoires, avec un large spectre d'effets qui induisent l'apoptose ou la nécrose. L'IL-6 module les actions pro- et anti-inflammatoires et induit la vasoconstriction et l'hypertension (Chen *et al.* 2010). Ces deux cytokines sont augmentées dans le sérum maternel des patientes atteintes de

prééclampsie, entraînant l'activation des lymphocytes T auxiliaires Th-1 et une réponse immunitaire exacerbée (L. M. Chen *et al.*, 2010b). Contrairement à IL-6 et au TNF, IL-10, qui est connue pour contrôler la réponse immunitaire et activer la réponse Th-2, est diminuée par l'H/R dans le STB (Makris *et al.*, 2006). Donc, on a pu vérifier la pertinence de notre modèle pour mimer *in vitro* la modulation des cytokines engendrées par la prééclampsie *in vivo*. De plus, la modulation des cytokines TNF, IL-6 et IL-10 causés par l'H/R ont été diminuée par un traitement avec la mélatonine à des taux similaires à ceux libéré par le STB en normoxie. Enfin, l'inhibition de la production de mélatonine endogène par l'inhibition de l'enzyme de synthèse AANAT par la stratégie de siARN a augmenté le taux d'IL-6 en normoxie et en H/R. Ce résultat suggère que la mélatonine endogène a un rôle inhibiteur sur l'expression ou sur la libération d'IL-6 au cours de la grossesse et que la mélatonine exogène pourrait être utilisée pour contrecarrer l'augmentation de cette cytokine observée dans les pathologies de grossesse, tel que la prééclampsie (**Figure 13**).

Nos résultats montrent que l'H/R a un effet pro-inflammatoire semblable au LPS utilisé dans notre modèle comme contrôle positif activateur d'inflammation. L'H/R comme le LPS active le facteur de transcription NFκB, l'activité autophagique et la mort cellulaire, tandis qu'il réduit la synthèse de mélatonine. Nous avons précédemment démontré une réduction des taux de mélatonine dans les placentas de grossesse compliquée par une prééclampsie comparés à ceux de grossesse normotensive, mais la raison de cette réduction demeure inconnue (Lanoix *et al*. 2012). Les résultats de la présente étude suggèrent que la diminution de mélatonine serait causée par l'activation de NFκB et de TNF par l'H/R. Dans ce sens, nous avons montré au **chapitre 3** que l'inhibition de NFκB, comme la mélatonine, entraîne une augmentation de l'activation de l'autophagie et une diminution de l'apoptose. Ceci suggère que la mélatonine agit comme un modulateur de l'inflammation dans le STB. En fait, des études ont montré qu'il existe un mécanisme de rétroaction (en général négatif) entre la mélatonine et le NFκB (Markus et al. 2017; Muxel et al. 2016; Franco and Markus 2014). Dans ce sens, la figure 7 du **chapitre 3** montre que l'inhibition de NFKB sous normoxie induit la phosphorylation de AANAT (forme active de l'enzyme). En H/R, cependant, l'inhibition de NFκB n'est pas capable de restaurer le taux de phosphorylation de AANAT, suggérant que d'autres facteurs pourraient être impliqués. Des études ont montré que le miR-325-3p régule à la baisse l'expression de AANAT en condition hypoxique. De plus, miR-325-3p est significativement augmenté dans les cellules endothéliales exposées au TNF (Chakraborty *et al.* 2015). Dans ce sens, nos résultats démontrent que, sous H/R, l'inhibition de NFκB est associée à une augmentation significative

de TNF. Ceci suggère qu'il existe un mécanisme de modulation du TNF sur l'AANAT, mais il reste à étudier s'il dépend du miR-325-3p.

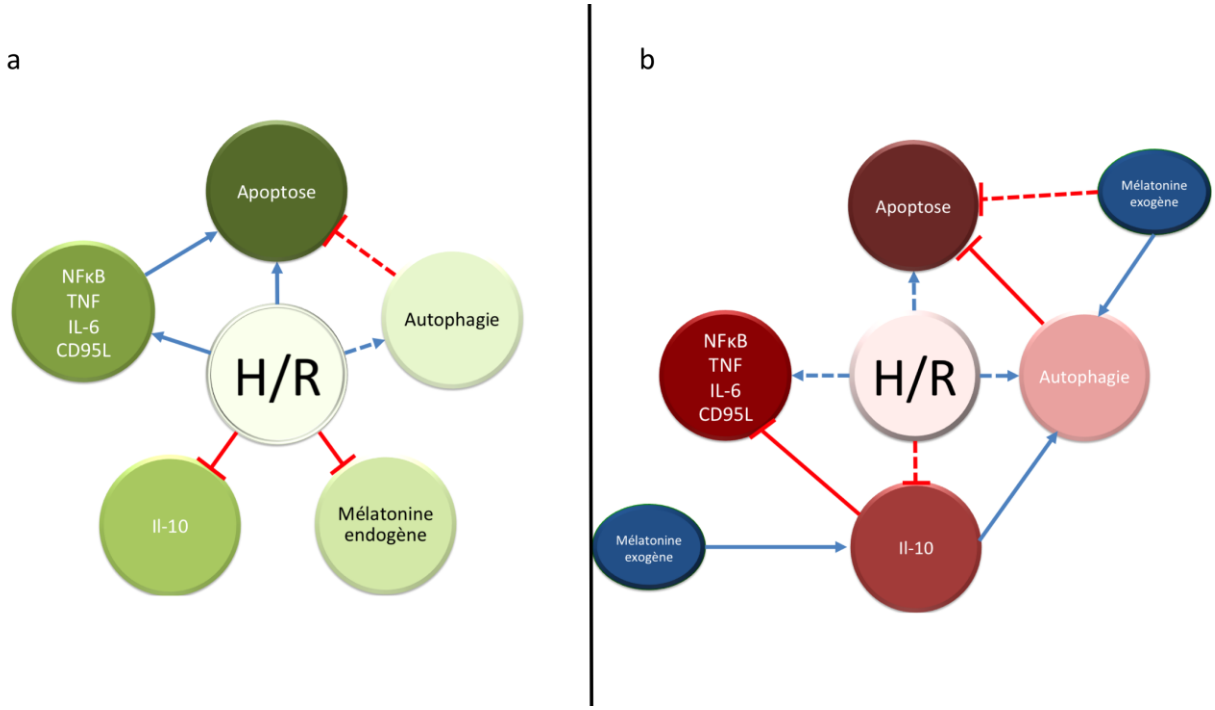

Figure 13: Résumé des mécanismes mis en jeux dans le rôle protecteur de la mélatonine dans le trophoblaste villeux primaire exposé à l'hypoxie/réoxygénation (H/R). (a) L'exposition à l'H/R inhibe la production endogène de mélatonine et la libération de l'interleukine (IL) -10, tandis qu'elle augmente les facteurs pro-inflammatoires et pro-apoptotiques (NFκB, IL-6, TNF et CD95 ligand (CD95L)) ainsi que l'apoptose. L'H/R induit l'activation de l'autophagie, cependant ce mécanisme de pro-survie n'est pas capable de sauver les primocultures de trophoblaste villeux de la mort cellulaire. (b) Le traitement par la mélatonine exogène augmente la libération d'IL-10, qui inhibe les réponses pro-inflammatoires causées par l'H/R et induit l'activation de l'autophagie, ce qui conduit à une réduction de l'apoptose.

Cette interrelation entre le système de la mélatonine et l'inflammation et les observations suivantes : (1) l'H/R induit l'apoptose extrinsèque et l'inflammation ; (2) la prééclampsie est liée à une tolérance immunitaire envers le placenta; et (3) les résultats du **chapitre 3** démontrant que la mélatonine agit comme anti-inflammatoire en situation d'H/R et donc active les voies en aval de l'inflammation, nous a conduits à la question de recherche suivante : **Est-ce que le système CD95 est impliqué dans les pathologies la grossesse associée avec une H/R placentaire**? CD95 régule l'activation immunitaire et la perturbation de ce système est liée à des maladies auto-immunes, telles que le lupus érythémateux disséminé, et à des pathologies de la grossesse, telles que les avortements spontanés (Darmochwal-Kolarz *et al.*, 2001, Ehrlich

et al., 2007). Pour répondre à cette question, nous avons choisi d'utiliser un modèle *ex vivo*, des explants de villosité du premier trimestre de la grossesse et à terme. Les explants du premier trimestre ont été obtenus à partir d'avortements électifs (9 à 13 semaines de grossesse). Les explants à terme (38-41 semaines de grossesse) ont été exposés à une H/R avec ou sans mélatonine 1 mM (voir *Materials and Methods,* **chapitre 5** pour la méthodologie détaillée).

Comme décrit dans l'**annexe 4**, l'apoptose contrôlée par le CD95 a une importance primordiale pour l'élimination des lymphocytes T autoréactifs impliqués dans les maladies auto-immunes (Krammer, 2000, Peter *et al.*, 1998). Le privilège immunitaire observé dans la grossesse est dépendant de la plasticité des lymphocytes T maternels. En outre, le CD95L placentaire inhibe les lymphocytes T maternels, ce qui confère le privilège immunitaire du placenta. L'équilibre Th-1/Th-2 avec une dominance Th-2 est nécessaire pour le bon déroulement de la grossesse. Cet équilibre est entre autres régulé par le TNF et l'interféron gamma (INFγ), deux cytokines Th-1, qui augmente l'expression du CD95 et l'apoptose contrôlée par CD95. D'un autre côté, l'IL-10 conduit à l'augmentation de l'expression du *FLICE inhibitory protein* (FLIP), qui bloque à son tour le signal apoptotique dans la primoculture de trophoblaste humain de premier trimestre (Saito *et al.* 2010). Les figures 1 et 2 du **chapitre 5** démontrent que l'H/R induit l'apoptose extrinsèque via la voie de signalisation du CD95 dans les explants de villosité placentaire à terme. En H/R, il existe une corrélation positive entre l'expression de CD95L et de cl-caspase 3, suggérant que l'H/R altère le privilège immunitaire placentaire. Pour la première fois, nous démontrons que la mélatonine restaure les taux de CD95L et de cl-caspase 3 aux taux observés en normoxie.

Malgré la similarité des résultats des **chapitres 3 et 5** concernant les actions de la mélatonine sur l'inflammation et l'apoptose, une limitation importante consiste à distinguer quelles cellules placentaires sont affectées par les conditions d'H/R et également par le traitement de la mélatonine dans les explants (**chapitre 5**). Le syncytiotrophoblaste a été le focus du **chapitre 3,** mais malgré son rôle important d'échange entre la mère et le système fœtoplacentaire, il n'est qu'un des nombreux types de cellules présentes dans le placenta humain. De plus, la majorité des résultats présentés ont été réalisés avec des placentas de grossesses à terme (38 à 42 semaines de grossesse), ce qui représente un moment unique et spécifique de la grossesse. Comme décrit par Ji et al. 2013 (Ji *et al.*, 2013), le placenta est un organe en constante évolution différentiation, avec des taux élevés de prolifération et de différenciation cellulaires tout au cours de la grossesse. La nécessité de la compréhension des évènements moléculaires liés au comportement des cellules placentaires au cours du premier et du deuxième trimestre est impérative pour améliorer les traitements prometteurs des troubles

de la grossesse par la mélatonine. Notre groupe a démontré que les enzymes de synthèse et les récepteurs de la mélatonine sont présents et actifs tout au cours de la grossesse normale (Soliman *et al.*, 2015) par contre, il n'y a aucune étude à ce jour qui a analyser les modulations du système mélatoninergique sous des conditions sous-optimales, comme l'H/R, au cours de la grossesse.

Les résultats préliminaires du **chapitre 5** suggèrent que, en parallèle à son action de protection de la mitochondrie, la mélatonine aurait un rôle inhibiteur de l'apoptose extrinsèque dans les cellules placentaires exposées à l'H/R. Par ailleurs, les résultats ouvrent une nouvelle avenue de recherche pour mieux comprendre le rôle de la mélatonine dans la grossesse et l'immunité. Il serait, entre autres, intéressant d'étudier la présence et le taux d'expression du récepteur *Decoy receptor 3* (DcR3), capable de lier et de neutraliser CD95L. DcR3 est un membre de la superfamille des récepteurs du TNF et qui inhibe l'interaction CD95/CD95L. Par conséquent, son expression pourrait exercer un mécanisme de protection contre les effets cytotoxiques de CD95L (Pitti *et al*. 1998). Enfin, l'étude des voies apoptotiques et non apoptotiques modulées par le ligand homotrimérique soluble CD95L (sCD95L) serait une avenue intéressante. La comparaison des effets de sCD95L dans les cellules BeWo, les vCTBs, le STB et les explants placentaires permettraient de comprendre le rôle de ce facteur dans un modèle de cancer, sur la différenciation du vCTB et sur les communications des différents types de cellules qui forment le placenta humain. Ces approches pourraient aider à valider le rôle possible du système CD95 dans la syncytialisation et sur le privilège immunitaire du placenta.

D'autre part, l**e chapitre 5** (figure 3) montre des taux plus élevés de CD95L et de caspase 3 clivée dans les explants du premier trimestre par rapport aux explants à terme. Ceci pourrait être lié à un renouvellement cellulaire plus fréquent, un processus physiologique dans les tissus en croissance et en multiplication, tel que le placenta au cours du premier trimestre (Jerzak *et al.* 2002). Cette différence pourrait également être liée à l'invasion trophoblastique, et ainsi une dérégulation du CD95 pourrait être associée à une augmentation du risque d'avortement spontané. Les Th-17 qui interviennent dans l'infiltration tissulaire et l'expression des cytokines pro-inflammatoires sont augmentés de façon importante dans l'utérus des avortements spontanés (Wang *et al.* 2010). Les cellules régulatrices T (Treg) contrôlent la tolérance immunitaire de la grossesse. Ce sous-ensemble de lymphocytes T est responsable du contrôle de la réponse immunitaire excessive par l'induction de l'IL-10 et le TGF-β. Dans la grossesse normale, le ratio Th-17/Treg se déplace en faveur de la voie anti-inflammatoire Treg (Figueiredo *et al.*, 2016). En revanche, dans les cas d'avortement spontané et de prééclampsie,

le ratio Th-17/Treg est déstabilisé avec une augmentation de Th-17 et des taux similaires de Treg dans le. Le sCD95L est capable d'induire le trafic de Th-17 dans les organes où le taux d'inflammation est élevé et induire l'activation de réponses pro-inflammatoires (Poissonnier *et al.*, 2016). Fait intéressant, les taux de cl-CD95L sont augmentés chez des cas d'avortement spontané ou de prééclampsie sévère, ce qui pourrait impliquer le CD95 comme un des facteurs impliqués dans les lésions du tissu placentaire.

6.3 Mélatonine : actions protectrices sur les cellules normales et toxiques sur les cellules tumorales

En somme, les résultats de cette thèse démontrent que notre modèle d'H/R conduit à la réduction de l'homéostasie des primocultures de STB et des explants de villosité isolés de placenta de grossesse normale à terme. En outre, l'H/R active l'autophagie, l'inflammation, l'apoptose et diminue la synthèse de mélatonine dans le STB. Nous avons également démontré que la mélatonine exogène a des actions protectrices qui augmentent de la survie cellulaire en condition d'H/R. Dans le **chapitre 2**, nous démontrons que sous l'H/R, la mélatonine restaure le taux des EROs à un taux basal sans affecter la différenciation des vCTB en STB. Dans le **chapitre 3**, nous avons montré que la mélatonine active significativement l'autophagie dans le STB sous H/R et ainsi réduit l'apoptose. De même, dans le **chapitre 4**, nous montrons que l'H/R induit l'activation de l'autophagie et du facteur de transcription Nrf2 dans le vCTB. Le traitement avec la mélatonine augmente l'activation de ces deux voies pro-survies pour protéger les cellules exposées à l'H/R. Enfin, dans les **chapitres 3** et **5**, nous avons observé une augmentation des événements pro-inflammatoires, entraînant la mort cellulaire suite à une H/R et qu'un traitement avec 1 mM mélatonine rétablit la balance inflammatoire vers un profil anti-inflammatoire, ce qui a conduit à la réduction de l'autophagie et de la mort cellulaire. Cette étude appuie l'intérêt croissant des scientifiques et cliniciens pour la mélatonine comme traitement potentiel dans les cas de maladie de la grossesse, comme la prééclampsie. Dans ce sens, Hobson et al., ont récemment publié des données cliniques sur les effets de la mélatonine dans le traitement de grossesse compliquée par une restriction de croissance intra-utérine ou par une prééclampsie. Ils ont montré que la mélatonine réduit le taux de prématurité et réduit la prise de médicaments antihypertenseurs, dans les grossesses compliquées par une prééclampsie. Le potentiel de la mélatonine comme traitement des troubles de la grossesse nécessite encore des investigations, mais est extrêmement prometteur (Hobson *et al.* 2018).

Contrairement à son action cytoprotectrice dans les cellules normales, la mélatonine est de plus en plus suggérée comme une molécule anti-tumorale (Haghi-Aminjan *et al*. 2018; Asghari *et al*. 2018). Dans ce sens, notre groupe a déjà montré que, dans les cellules BeWo, la mélatonine perturbe le potentiel des membranes mitochondriales et active l'apoptose intrinsèque, tandis qu'elle diminue l'activation de l'apoptose dans le trophoblaste primaire (Lanoix *et al.* 2013). Dans la présente étude, les résultats présentés dans le **chapitre 4** montrent que la mélatonine est cytotoxique dans les cellules de choriocarcinome BeWo et cytoprotectrice dans les vCTB primaires. Les cellules cancéreuses, y compris les cellules BeWo, sont résistantes aux modulations d'oxygénation. Une explication pour ce phénomène serait due à un taux basal plus élevé d'autophagie. En accord avec cette hypothèse nous avons montré dans les BeWo que l'H/R n'augmente pas l'activation du facteur LC3B, responsable pour la liaison du *cargo* autophagique à la membrane de l'autophagasome. De plus, dans les cellules BeWo la mélatonine réduit l'activation de l'autophagie et du facteur de transcription Nrf2 en condition d'H/R, tandis qu'inversement dans les vCTBs cette indolamine active l'autophagie et le Nrf2. Ces résultats sont en accord avec la littérature qui montre que la mélatonine possède des actions cytotoxiques dans les cellules tumorales (voir l'article de revue (Reiter *et al.* 2017)). Cette différence d'effets de la mélatonine dans les cellules normales et cancéreuses est encore mal comprise. Il a été démontré que la mélatonine modifie le profil d'expression des miARN et la méthylation de l'ADN dans des cellules de cancer du sein. Au contraire, la mélatonine réduit la formation de 8-hydroxy-désoxyguanosine par des actions antioxydantes pour aider à éviter l'échec de la réparation de l'ADN dans les îles CpG dans les neurones normales (Hardeland, 2014, Hardeland *et al.*, 2011, D. Y. Li *et al.*, 2013). Il serait intéressant de comparer les modulations épigénétiques engendrées par la mélatonine dans les BeWo et dans les cellules primaires trophoblastiques. (Lopez-Burillo et al. 2003). Comme décrit par Hardeland, la mélatonine agit comme un modulateur épigénétique (Hardeland, 2014), mais les mécanismes impliqués et si ce phénomène existe aussi au niveau placentaire n'a jamais été étudié (Figure 14).

Malgré que cette étude permette de mieux comprendre le rôle et le mécanisme d'action de la mélatonine dans les cellules placentaires normales et tumorales, il reste encore beaucoup à étudier, dont, entre autres, le rôle potentiel des récepteurs MT1 et MT2. Comme décrit dans le chapitre 3, le luzindole, un inhibiteur des récepteurs MT1 et MT2, n'a pas d'effet sur la libération de TNF (Data not shown 1) et d'IL-10 (Data not shown 2) par rapport aux cellules exposées à la mélatonine. Ceci suggère que les effets de la mélatonine sur le TNF et l'IL-10 ne sont pas dépendants de ses récepteurs membranaires (MT1 et MT2). Cependant, il convient de

noter que le luzindole est également un antioxydant (Mathes *et al.*, 2008, Requintina *et al.*, 2007) et peut donc mimer les effets antioxydant récepteurs-indépendants (direct) de la mélatonine. En ce qui concerne les cellules tumorales, comme décrit dans la revue de Reiter et al. 2017 (Reiter *et al.*, 2017), les récepteurs de la mélatonine seraient des médiateurs importants des effets cytotoxiques de la mélatonine dans des modèles comme le cancer de foie et le glioblastome. L'interférence de l'expression MT1 et MT2 aurait apporté une compréhension plus large des actions de la mélatonine récepteurs-dépendants et -indépendantes. Contrairement à d'autres lignées de cellules tumorales, les cellules BeWo présentent un taux de prolifération plutôt lent . En plus, les trophoblastes villeux primaires humains ne peuvent pas subir de mitose *in vitro* et entrent en apoptose après 4 jours de culture donc le taux de transfection est faible dans ces deux modèles. Finalement, une autre limitation est que nous n'avons étudié qu'une lignée cellulaire comme modèle de tumeur trophoblastique. Il serait important valider les données du **chapitre 4** sur des trophoblastes primaires issus de tumeurs placentaire.

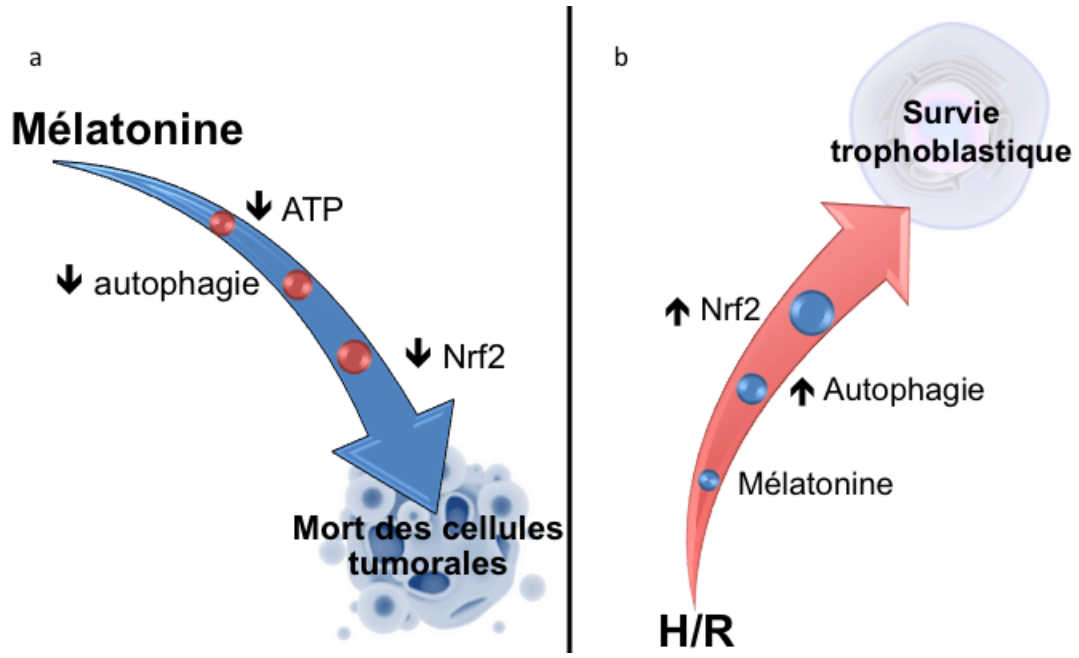

Figure 14: La mélatonine agit différemment dans les cellules placentaires normales et cancéreuses. (a) Dans la lignée de cellules de choriocarcinome BeWo, la mélatonine augmente la phosphorylation de la *5' adenosine monophosphate-activated protein kinase* (AMPK), en raison de la réduction des taux d'ATP. La mélatonine inhibe l'activité autophagique et l'activation du facteur de transcription *Nuclear factor (erythroid-derived 2)-like 2* (Nrf2), conduisant ainsi les cellules à la mort cellulaire. (b) Dans les primocultures de cytotrophoblastes villeux issues de placentas de grossesses normales à terme, l'H/R perturbe l'homéostasie cellulaire. Le traitement à la mélatonine induit l'activation

de deux mécanismes de pro-survie : l'autophagie et le Nrf2, et rétablit l'homéostasie et maintien la survie cellulaire.

6.4 Conclusions générales

Au cours des 5 dernières années, l'intérêt de la communauté scientifique pour les rôles protecteurs de la santé materno-fœtale attribués à la mélatonine s'est accru exponentiellement. L'effet protecteur de la mélatonine dans le placenta a été décrit précédemment, mais les mécanismes d'action mise en jeux demeurent mal compris. Les résultats de cette thèse apportent une meilleure compréhension des effets de la mélatonine sur l'autophagie et l'inflammation, des voies activées dans les cellules placentaires en situation d'H/R associés entre autres aux grossesses compliquées par une prééclampsie. Les données obtenues durant mon doctorat apportent une meilleure compréhension de la mélatonine dans le contexte de la grossesse comme une option sécuritaire et efficace contre des maladies et troubles de la grossesse. De plus, nos résultats montrent différents effets de la mélatonine selon le modèle expérimental : cellules de choriocarcinome placentaire, cellules primaires placentaires et tissus *ex vivo* placentaires. Il reste cependant encore des questions sans réponse concernant les effets de la mélatonine sur le placenta et sur les échanges entre les cellules maternelles et les cellules placentaires. Des études intégratives et translationnelles sur les effets de la mélatonine dans le placenta et conséquemment sur la santé maternofœtale sont encore nécessaires pour valider la mélatonine comme agent protecteur de l'homéostasie cellulaire tant en situation tumorale (cytotoxique), que de stress cellulaire (cytoprotectrice).

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ANNEXE 1 – CHAPITRE DE LIVRE : IN VITRO INDUCTION OF HYPOXIA/REOXYGENATION ON PLACENTAL CELLS: A SUITABLE MODEL FOR UNDERSTANDING PLACENTAL DISEASES

Contribution de l'étudiant

Lucas Sagrillo-Fagundes a participé à la validation de la méthode, réalisé les expériences et analysé les résultats. Il a également rédigé le chapitre du livre et effectué les corrections demandées par les évaluateurs.

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Chapter 21

In Vitro Induction of Hypoxia/Reoxygenation on Placental Cells: A Suitable Model for Understanding Placental Diseases

Lucas Sagrillo-Fagundes, Laetitia Laurent, Josianne Bienvenue-Pariseault, and Cathy Vaillancourt

Abstract

Lack of blood flow and aberrant levels of oxygenation in placentas are recurrent in pregnancy diseases, such as preeclampsia. These alterations generate situations of hypoxia and hypoxia/reoxygenation (H/R) and consequent oxidative stress, increased cell death, and inflammation in trophoblasts. The models used to understand the effects of hypoxia and H/R on trophoblasts require a rather big structure. This chapter describes the details of a suitable and reasonable approach with hypoxia chambers to expose human placental trophoblasts to variable conditions of oxygenation.

Key words Normoxia, Hypoxia, Hypoxia/reoxygenation, Villous trophoblast, Extravillous trophoblast, Syncytiotrophoblast

1 Introduction

Across the first trimester of pregnancy, hypoxia has a key role in the uterine invasion. This stimulus is essential for the invasion of extravillous trophoblasts (evTB) and the achievement of endovascular trophoblasts toward the enlargement of uteroplacental arteries and consequent increased placental blood flow. Low concentrations of oxygen (15–20 mmHg or <2% O_2) during the placentation are gradually increased, and the end of the pregnancy is coincident with relatively high levels of oxygen (55–60 mm Hg or <8% O_2) [1, 2]. This increase is tightly regulated and is correlated with successful pregnancy outcomes. The lack of regulation in this process leads to alterations such as preeclampsia and intrauterine growth restriction (IUGR) [3]. The etiology of preeclampsia is still a source of debates and some epidemiological studies suggest that it has a genetic and immunological origin. Regardless of its origin, the effects of preeclampsia are well characterized with several

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effects for maternal and fetal health according to its intensity and mainly with its onset point [4, 5]. Just as preeclampsia, IUGR is a major morbidity during the pregnancy. Both of these diseases are associated with an abnormal first-trimester trophoblast differentiation. This happening is related with a shallow placental invasion and decreased remodeling of maternal spiral arteries and a consequent failure at the uteroplacental perfusion. Reduced perfusion leads to episodes of hypoxia or hypoxia/reoxygenation (H/R), correlated with inflammatory processes, aberrant cell death, and oxidative stress in trophoblasts [2, 5]. The comprehension of the effect of aberrant oxygenation during the pregnancy has been a source of experimental and epidemiological studies.

Human placenta is classified as type haemomonochorial, only present in superior apes. In this kind of placenta, syncytiotrophoblast has a direct contact with the maternal blood and is able to produce the hormone human chorionic gonadotropin (hCG). The unique architecture of human placenta is a challenge for animal models as it varies drastically even in the order of primates. Usual animal models (e.g., rats and mice) can only reproduce some characteristics of preeclampsia under pharmacological induction. Only members of the family Homininae (humans, chimpanzees, and gorillas) have a similar pattern of deep evTB invasion and spiral artery remodeling [6, 7]. In this context, placental cell lines, primary isolated cytotrophoblast cells, and even placental explants have been shown as suitable models for the comprehension of H/R to the placental homeostasis. Moreover, these models have been used to understand the molecular events involving preeclampsia and IUGR [8–10]. A great variety of approaches have been developed to reproduce properly the placental environment under hypoxic conditions along the last decades with results with increasing levels of comprehension and complexity.

The procedure detailed in this chapter has been used in several approaches during the last years with consistent results and reproducibility and has a relatively affordable cost.

2 Materials

1. Modular incubator chamber (a set of two chambers is necessary for simultaneous induction of normoxia, hypoxia, and H/R).
2. Single flow meter.
3. 50 mm In-Line Filter (PTFE, pore: 1.0 μm).
4. Pro Star Gas Regulator.
5. Gas hose class VI clear 5/16 (three pieces with ~0.5 m).
6. 17 mm adjustable gas hose clamp.

7. Gas cylinder size K:
   (a) Normoxia (third trimester's composition: 5% CO_2, 8% O_2, Bal. N_2).
   (b) Normoxia (first trimester's composition: 5% CO_2, 2% O_2, Bal. N_2).
   (c) Hypoxia (5% CO_2, 0.5% O_2, Bal. N_2).
8. Oxygen microelectrode.
9. Oxygen adapter.
10. pH-mV meter.

3 Methods

3.1 Hypoxia Chamber Assembling with the Flow Meter and a Cylinder of Gas (Fig. 1)

1. Attach a hose (*5*) with a clamp (*6*) to the gas cylinder (*7*) (*see* **Note 1**).
2. Insert the 1 μm pore size filter (*3*) between the hose attached to the cylinder (*5a*) and the inlet hose (*5b*) of the flow meter (*2*).
3. Connect the outlet hose (*5c*) of the flow meter to the inlet port of the hypoxia chamber (*1*).
4. Open the outlet port of the hypoxia chamber.

3.2 Hypoxia Chamber Operation (Fig. 1)

1. Inside a laminar flow hood, open the chamber and place a petri dish with sterile water to avoid dehumidification.
2. Place the plates or cell culture flasks previously prepared on the superior levels of the chamber.

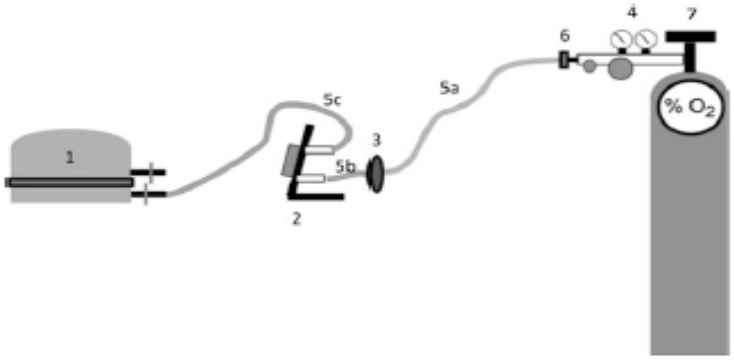


Fig. 1 Schematic representation of the components of a hypoxia/reoxygenation chamber. Hypoxia chamber and gas cylinder assembling: (*1*) modular incubator chamber; (*2*) flow meter; (*3*) inlet filter; (*4*) gas regulator; (*5a*) cylinder gas hose; (*5b*) inlet hose; (*5c*) outlet hose; (*6*) gas hose clamp; (*7*) gas cylinder (Modified from Sagrillo-Fagundes et al. 2016 [12])

3. Close the chamber inside the hood.
4. Outside the hood, attach the chamber (inlet port) by the gas hose (*5c*) to the set of source of gas (*7*) as described in Subheading 3.1. Open both inlet and outlet ports of the chamber. At this moment the gas regulator (*4*) should remain closed.
5. Carefully open the valve of the gas regulator (*4*) (*see* **Note 2**). The flow meter (*2*) will indicate the amount of air (L/min) that is accessing the chamber. A flush of 4 min with an amount of 25 L/min is necessary to completely change the gas inside the chamber.
6. After the intended time of air flow, close the gas regulator (*4*) and then close the inlet and outlet ports of the chamber.
7. Unplug the flow meter outlet hose (*5c*) from the inlet port of the chamber.
8. Set the chamber in a cell incubator at 37 °C.
9. The chamber must be filled with gas again 1 h afterward. The purpose of this last purge is to remove the air presumably present in the plates, flasks, and dissolved in the culture medium.
10. Repeat **steps 1–9** for chambers comprising other gas configurations.

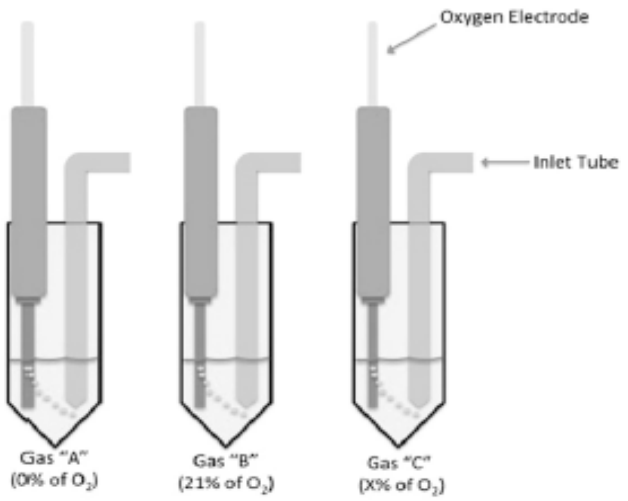
3.3 Confirmatory Oxygen Measurement (Fig. 2)

1. To confirm the concentration of oxygen in the cell medium kept inside the chamber, it is necessary to use an oxygen electrode hooked up to an Oxygen Adapter. The oxygen electrode must be connected to a voltmeter or an mvMeter.
2. To create a calibration curve, expose cell media to a range of gas mixtures containing known concentrations of oxygen (e.g., expose to gas mixtures with 0% of oxygen and to 21% of oxygen). After acquiring a stable value at each point of the curve, the electrode is ready to be introduced in the medium exposed in the chamber. As the concentration of oxygen is dependent upon the depth within the medium, all the measurements must be held at the same depth [11, 12].

3.4 Induction of Normoxia, Hypoxia, or H/R: Placental Studies

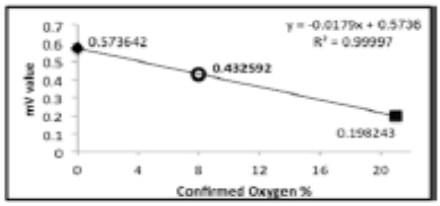
1. After seeding placental cell lines, primary culture of trophoblasts or explants in appropriate flasks, plates, or petri dish, inductions can be performed as necessary (*see* **Note 3**), and every 24 h cells are reexposed to the desired gas mixture to reproduce a specific condition (Fig. 3a).
2. The physiological condition that evTB are exposed during the first trimester is also reproducible with modular incubator chambers [13, 14] (Fig. 3b).

A.



B.

	% of O_2	mV value	Confirmed % of O_2
◆	0%	0.573642	0%
○	?%	**0.432592**	**7.879888%**
■	21%	0.198243	21%



C.

Y= ax + b ?%= (0.432592 – 0.573642)/-0.0179

a= slope ?%= (-0.14105)/-0.0179

b= 0% mV value ?%= 7.879888% of O_2

Fig. 2 Representation of dissolved oxygen concentration. (**a**) Confirmation of oxygen concentration in a cell medium using other solutions with known concentration of oxygen. (**b**) The relative values obtained in the solutions "A" and "B" are plotted in a graphic of linear function to confirm the oxygen concentration in the solution "C." (**c**) Data obtained in the graphic are applied to a linear function determination to confirm the oxygen concentration in solution "C" (Modified from Sagrillo-Fagundes et al. 2016 [12])

4 Notes

1. During the assembling of the components, all valves must be closed.
2. Caution is advised for injecting gas inside the chambers due to their relative small size.

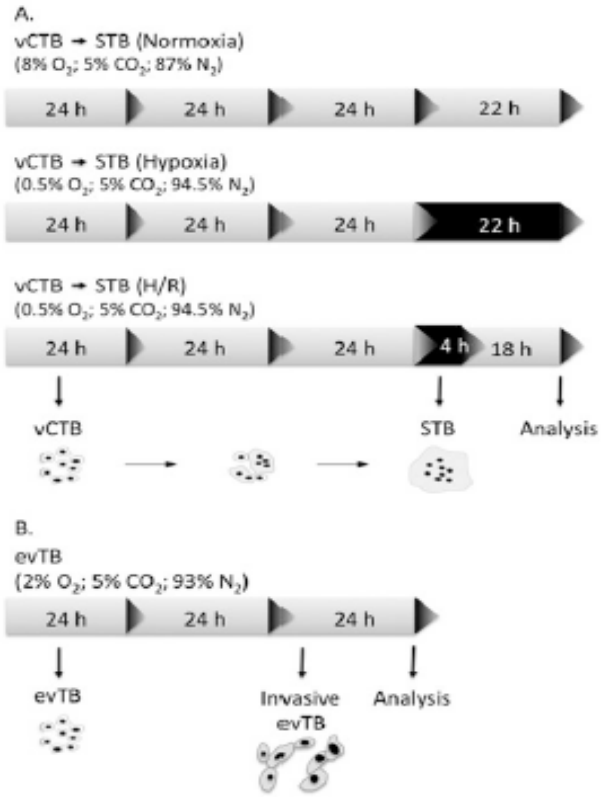


Fig. 3 Generic model of cell culture in modular incubator chamber. (**a**) Normoxia, hypoxia, and hypoxia/reoxygenation (H/R) are adequate methods to study pathological conditions in STB (*see* **Note 4**). Every 24 h (*black triangle*), gas should be renewed. Under hypoxic conditions, STB (from 72 h onward) are exposed continuously to 0.5% of O_2. Under H/R, STB are exposed during 4 h to hypoxia and then are reexposed to normoxia (8% O_2). (**b**) The rate of oxygenation that evTB are exposed during the first trimester of pregnancy is obtained using the adequate gas mixture with the modular incubator chamber. *vCTB* villous cytotrophoblast, *STB* syncytiotrophoblast, *evTB* extravillous trophoblasts

3. Incubation chambers allow performing any protocol of treatment/induction. However, every time that a chamber is opened to treat or change the medium of cells, the gas mixture must be reflushed as described in Subheading 3.2.
4. Figure 3a reproduces a generic protocol of hypoxia induction for STB. To induce the same conditions for vCTB, the time of incubation should be reduced to a maximum of 48 h [12].

Acknowledgments

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ANNEXE 2 – ARTICLE DE REVISION : MELATONIN IN PREGNANCY: EFFECTS ON BRAIN DEVELOPMENT AND CNS PROGRAMMING DISORDERS.

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Lucas Sagrillo-Fagundes a participé à la validation des sujets de l'article. Il a également rédigé l'article et participé au choix du journal de publication et effectué les corrections demandées par les évaluateurs.

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Melatonin in Pregnancy: Effects on Brain Development and CNS Programming Disorders

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Cathy Vaillancourt

Abstract: Melatonin is an important neuroprotective factor and its receptors are expressed in the fetal brain. During normal pregnancy, maternal melatonin level increases progressively until term and is highly transferred to the fetus, with an important role in brain formation and differentiation. Maternal melatonin provides the first circadian signal to the fetus. This indolamine is also produced *de novo* and plays a protective role in the human placenta. In pregnancy disorders, both maternal and placental melatonin levels are decreased. Alteration in maternal melatonin level has been associated with disrupted brain programming with long-term effects. Melatonin has strong antioxidant protective effects directly and indirectly via the activation of its receptors. The fetal brain is highly susceptible to oxygenation variation and oxidative stress that can lead to neuronal development disruption. Based on that, several approaches have been tested as a treatment in case of pregnancy disorders and melatonin, through its neuroprotective effect, has been recently accepted against fetal brain injury. This review provides an overview about the protective effects of melatonin during pregnancy and on fetal brain development.

Keywords: Placenta, melatonin, preterm birth, neurogenesis disorders, neuroprotection, neural tube, fetus.

INTRODUCTION

Melatonin is produced by the pineal gland and is known as a hormone responsible for the circadian rhythm regulation. However, it is now well established that virtually all tissues and cells produce this indolamine. Its importance involves not only endocrine actions; melatonin has also paracrine, intracrine, and autocrine functions due to its physicochemical features, such as amphiphilic profile and small size. Melatonin plays a crucial role in pregnancy and fetal development, and has been shown to be produced by and protect the placenta [1-3].

Neurogenesis is activated in the early stages of pregnancy and is completely susceptible to exogenous factors that can induce or inhibit neuronal differentiation. Melatonin has been shown as an important factor for brain protection and its receptors are present in the fetal brain. Its role is related to its antioxidant properties, activation of intracellular protection pathways via its specific receptors, and epigenetic modulation. The protective effects of melatonin are highly related with fetal brain programming and therefore a disruption in maternal melatonin production might affect the fetal programming with long-term consequences [4]. Indeed, maternal melatonin levels could be modulated by mother's lifestyle, nutrition and exposure to chemical endocrine disruptors.

Pregnancy complications are associated with placental dysfunction. Preeclampsia, intrauterine growth restriction (IUGR), pre-term birth, and gestational diabetes are examples of pregnancy disorders that lead to altered fetal development with consequences on brain formation [5]. Pregnancy complications generate homeostasis disruption, not only in the placenta but also in the fetus with effects on its formation and programming [6].

Melatonin is an important factor for successful pregnancies positively affecting maternal-fetal homeostasis and fetal brain development [7]. Melatonin has been proposed as a possible treatment for several pregnancy disorders. Recently, this indolamine has been suggested as a neuroprotective agent for fetal brain. These days several clinical trials worldwide use melatonin as the main therapy against perinatal brain damage [8]. In this review we will present the wide effects of melatonin throughout pregnancy focusing on its effects on brain programming.

MELATONIN

About its discovery, melatonin was exclusively considered as the chief product of the pineal gland that regulates the circadian and circannual cycles. Synthesis of melatonin is carried out from the essential amino acid L-tryptophan. Melatonin rhythm is generated by the suprachiasmatic nucleus (SCN) in the hypothalamus and is highly inhibited by the quantity of luminosity to which SCN neurons are exposed via the retina [9]. Further studies revealed that melatonin is also produced by and present in various tissues and cells in the human body such as skin, retina, gastrointestinal tract, ovary, amniotic fluid and placenta [2, 10-13]. Melatonin's role in sustaining cell viability is mediated via receptor dependent and independent activities. Melatonin has both hydrophilic and lipophilic characteristics. This explains the presence of high levels of melatonin in all subcellular components including the nucleus, the cytoplasm, and the mitochondrion. This indolamine readily crosses the morphological barriers including the placental and blood brain barrier [14-18].

Melatonin is a potent antioxidant with higher activity compared to other antioxidants such as vitamin C and vitamin E [19]. Its superiority is due to its antioxidant metabolites cascade. When melatonin interacts with free radicals for their neutralization, a number of melatonin metabolites are produced such as N1-acetyl-5-methoxykynuramine (AMK). Interestingly, melatonin metabolites also have antioxidant properties and some of them may be more potent than melatonin itself [1, 3, 17]. Melatonin has also antioxidant properties through receptor dependent activity, as it upregulates the expression and activity of antioxidant enzymes. This action is mediated via the upregulation of transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and by epigenetic mecha-

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[$]Both authors (LSF, EMAS) contributed equally to this review.

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nism [20-22]. Melatonin also possesses anti-inflammatory properties as it modulates and affects the activity of the inducible form of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [23].

Two membrane G protein coupled receptors (GPCRs), MT1 (Mel1a) and MT2 (Mel1b), have been identified as able to bind melatonin. Melatonin interaction with the nuclear receptor family ROR/RZR (retinoid orphan receptor / retinoid Z receptor) has been also described [24, 25]. Despite the affinity of ROR/RZR for melatonin, there is still a controversy as to whether melatonin is the native ligand [24, 26, 27]. MT1 and MT2 receptors are widely distributed and have been found in most human tissues [reviewed in [15)]. Among them, MT1, MT2 and RORα (retinoid orphan receptor alpha) are expressed in primary cell culture of placental trophoblast and in two choriocarcinoma cell lines (JEG-3 and BeWo) [2, 28, 29]. In human fetal brain, melatonin receptors are also present and active starting at the mid-gestation [30, 31]. Calmodulin and MT3 (also known as Quinone reductase II) are other putative cytosolic melatonin receptors, however both have lower affinity to melatonin, if compared to MT1 and MT2 [32, 33].

Melatonin and Normal Pregnancy

Pregnancy is a complex physiological state with well-regulated and important maternal adaptations (e.g. increased metabolism and oxygen consumption) which are crucial for a normal pregnancy and fetal development [34]. Tryptophan derivatives, such as serotonin and melatonin, have increased levels during pregnancy and, interestingly, have been described as modulators of fetal development, such as brain differentiation and neurogenesis [2, 26, 35, 36]. During normal pregnancy, maternal circulating melatonin levels progressively increase after 24 weeks of gestation, with a maximum peak at 32 weeks then returning to basal levels after delivery. Pregnant women therefore have higher plasma melatonin concentrations compared to non-pregnant women [18, 37]. The daily rhythmicity provided by maternal pineal synthesis remains, but the basal plasma levels are increased over the pregnancy. The increased melatonin levels observed during pregnancy appear to come from placental production (Fig. **1**). The concentration of melatonin in human placental tissues is 6 to 7-fold higher than in the plasma [2, 29]. Moreover, melatonin levels return to non-pregnant levels after placenta delivery and also twin pregnancies showed higher melatonin levels compared to single pregnancies.

In accord with a placental source of melatonin, the presence and activity of the melatoninergic machinery (synthetizing enzymes and specific receptors) as well as *de novo* production was shown in trophoblasts from term placentas [2]. Melatonin in term trophoblastic cells has been shown to play an important protective role to the placental-fetal unit [reviewed in [26]. Recently, Soliman *et al.*, demonstrated that melatonin receptors and synthetizing enzymes are expressed in human placenta throughout pregnancy. The melatonin-synthetizing enzymes showed maximum expression in the third trimester, suggesting a continuous melatonin production during pregnancy with a peak in the late pregnancy [38]. These findings are consistent with data demonstrating high melatonin levels in maternal serum during pregnancy, also reaching a peak at the end of pregnancy then returning to non-pregnant levels after delivery [37-40]. The maximal production and expression of melatonin syn-

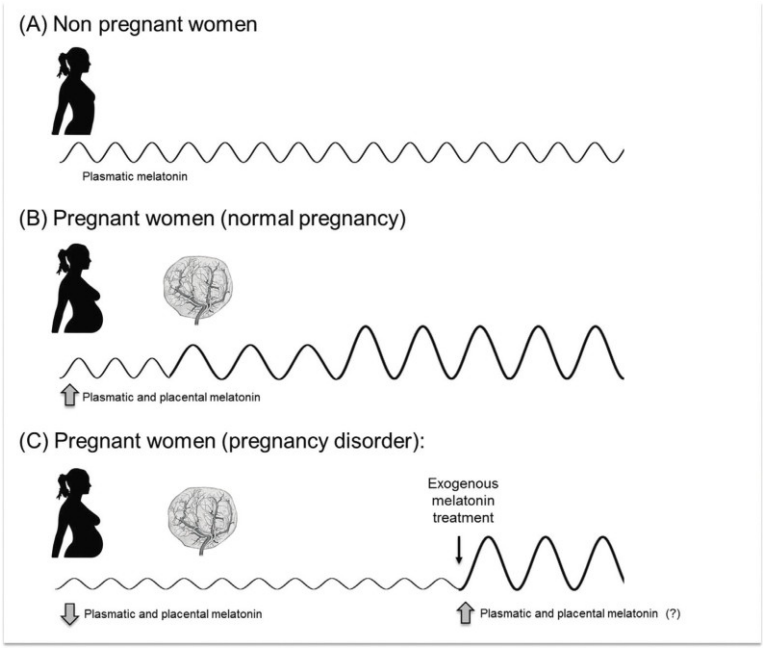

Fig. (1). Melatonin levels in pregnant and non-pregnant women. A) Non pregnant women have circadian production of plasmatic melatonin by pineal gland, with highest levels observed at night. (B) Pregnant women with normal pregnancies. Maternal plasmatic level of melatonin increases progressively until term and the circadian rhythmicity is maintained. Plasmatic melatonin levels increase probably due to non-circadian placental melatonin production. C) Pregnant women with pregnancy disorders, such as preeclampsia and intrauterine growth restriction (IUGR). Decreased levels of plasmatic melatonin are observed, probably due to alteration in placental melatonin production. Exogenous melatonin treatment could increase maternal plasmatic concentration of melatonin, hence increase fetal melatonin levels.

thetizing enzymes in the third trimester is an evidence that placental melatonin might be involved in the parturition process. Oxytocin and melatonin share the same signaling pathway to induce myometrium contractility [41]. Indeed, melatonin has been suggested to synchronize and facilitate parturition while alleviating the associated pain. MT1 and MT2 are expressed in uterine myometrium and pineal melatonin has been shown to control the normal time of parturition [42, 43]. An increased expression of MT1 was also observed during the first trimester compared to term placenta. This is coherent with the findings in embryo cells, corroborating the homology of primitive trophoblasts and embryoblasts [32]. Melatonin has also been described in several animal models of intrauterine growth restriction as able to increase the quantity of blood in the uterus and placenta. The supplementation of melatonin is also able to improve the absorption of complex types of amino acids even during maternal dietary restriction [44-46]. Different animal models have been used to understand the relationship between uterine blood-flow and placental vascularity during compromised pregnancies [47].

In this context, alterations in maternal or placental melatonin production might alter fetal melatonin levels and thus gene expression in the fetal central nervous system [48-50]. In rats, melatonin has been shown to protect the brain against oxidative stress, lipid peroxidation and DNA damage caused by ischemia and reperfusion during the embryonic period [50]. In pregnant rats, pinealectomy triggered maternal circadian cycle suppression, leading to behavioral alterations in newborns, which was restored after the induction of melatonin [48, 51]. Taken together, data from others and our group suggest a crucial role of melatonin and its receptors in placental health, and by consequence in pregnancy well-being and fetal development.

Melatonin and Pregnancy Disorders

Placental dysfunctions result in pregnancy pathologies such as preterm birth, gestational diabetes, intrauterine growth restriction and preeclampsia [52, 53]. Lanoix *et al*. have showed that the production of placental melatonin during pregnancy abnormalities is compromised due to breakdown of the synthetizing machinery of melatonin and decreased expression of its receptors (Fig. **2**) [54]. Consequently, exogenous melatonin has been suggested and tested in clinical trials as a treatment of pregnancy disorders [3, 18, 48, 54-57].

Placental programming is involved in fetal programming and is related with cardiovascular, metabolic and mental disorders during maturity [58, 59]. Neurodevelopmental disorders have been associated with complications during gestation with origin in early stages of pregnancy [60]. Preeclampsia and IUGR have been shown as a strong risk factor for the development of schizophrenia. Indeed,

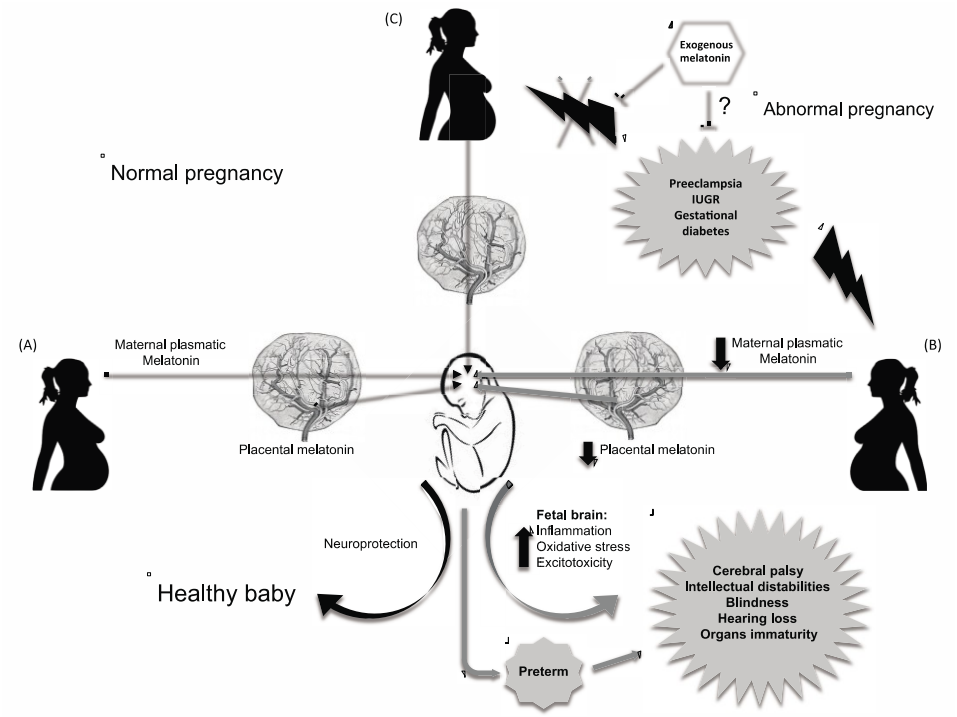

Fig. (2). Effects of melatonin on fetal brain development. **A**) Normal pregnancies show increased concentration of plasmatic melatonin over the time. Melatonin (placental and maternal) reaches fetal brain and, via specific receptors, protects and modulates neuronal replication and differentiation. **B**) Pregnancy disorders, such as preeclampsia, intrauterine growth restriction (IUGR) and gestational diabetes are associated with increased oxidative stress, which affects negatively the entire maternal-placental-fetal unit, causing increased oxidative stress, inflammation, and excitotoxicity in fetal brain, and also preterm birth, leading to long term brain disabilities. These pregnancy diseases are correlated with lower maternal plasmatic levels of melatonin and lower placental melatonin production. **C**) Recent clinical trials have proposed melatonin as a treatment against pregnancy diseases and as method to protect fetal brain.

even the arising of genetic forms of schizophrenia is modulated by the environment and lack of oxygen [61-64].

Preeclampsia affects around 10% of all pregnancies worldwide and is the major cause of maternal and perinatal morbidity and mortality [65]. This disease is characterized by hypertension and proteinuria after 20 weeks of gestation [66, 67]. The etiology remains unknown, however, problems during early stages of placentation result in vasoconstriction, placental hypoxia and inflammation leading to the clinical symptoms of the disease [68-70]. The altered blood flow of the placenta in preeclampsia is the major cause of premature birth and IUGR, as it results in oxygenation problems and insufficient nutrient supply to the fetus [71]. Preeclampsia is the obstetric complication with highest correlation with neurodevelopmental problems and schizophrenia [72]. Interestingly, maternal and placental levels of melatonin are decreased in pregnancy complicated by preeclampsia [18, 54, 73].

Intrauterine growth restriction is a condition characterized by lack of trophoblast invasion and consequent alteration of uteroplacental arteries formation, leading to fetal growth reduction [74]. This disorder affects approximately 10% of all pregnancies and is the second cause of perinatal morbimortality, followed by preterm birth [75]. Intrauterine growth restriction affects the development of several organs and systems and is associated with neurodevelopmental problems and other long-term complications [76, 77]. Intrauterine growth restriction and preeclampsia are intimately related: both are triggered by impaired placentation and uterine invasion during the first weeks of pregnancy; and, as elegantly demonstrated by Nakamura *et al*., lead to reduced maternal melatonin [18]. This condition is also associated with a reduction of melatonin and 6-sulphatoxymelatonin secretions in infants [78]. Recent studies have proposed the use of melatonin as an antioxidant therapy for intrauterine growth restriction. Based on experimental evidences, Alers *et al*. have been performing a phase I clinical trial to evaluate the benefits of maternal oral administration of melatonin in pregnancies affected by intrauterine growth restriction [79].

Gestational diabetes mellitus is a complication that affects 7 to 14% of all pregnancies [80] with increasingly incidence worldwide [81]. Gestational diabetes mellitus appears in the second and third trimesters of pregnancy and disappears after delivery. It is characterized by insulin resistance combined with hyperglycemia, endothelial dysfunction, and abnormal regulation of vascular tone [77, 82]. Its pathophysiology is not well understood and it has been suggested that a decrease in placental exchanges and hypoxia may contribute to its development [83]. Pregnancies complicated by gestational diabetes are commonly associated with adverse newborn outcomes, such as preterm birth, macrosomia, congenital malformations, fetal respiratory distress, and perinatal mortality [53, 84, 85]. Recently, epidemiological studies have reported that genetic variants of the gene that encodes MT2 protein are strongly associated with gestational diabetes mellitus in different populations [86-88]. Melatonin is also linked to glucose metabolism protecting B-pancreatic cells and reducing blood glucose levels in rats [89-91]. Evidences suggest that melatonin could reduce the effects of hyperglycemia, but its effect on gestational diabetes mellitus is not well elucidated [92]. Hyperglycemia causes excessive oxidative stress, protein glycation, inflammation, and is strongly associated with the progression of fetal-placental vascular complications [93-95. Moreover, gestational diabetes mellitus is linked with lower expression of the synthetizing enzymes and receptors of melatonin in placental tissue, a situation that affects even more the placental-fetal oxidative balance (personal unpublished data). Like placental alterations, maternal hyperglycemia has well-described behavioral effects in the newborns. These alterations have a negative correlation with cognitive performance, long-term behavioral alterations, and can even lead to neurological affections, such as schizophrenia [96-99].

MELATONIN AND FETAL BRAIN DEVELOPMENT

The formation of the brain begins during the early steps of pregnancy in human. About the third week of pregnancy, cells are already differentiated in three major phenotypes: ectoderm, mesoderm and endoderm. Endoderm cells are the origin of the neuronal cells that form the central and peripheral nervous system. Neuronal cells from endoderm form the neural tube that is the major organ of the embryo during the first weeks of pregnancy [100, 101]. Several kinds of cell differentiation are highly activated during brain development, such as neuronal migration, glial cell differentiation, neuronal segmentation, myelination and synaptogenesis [102-105]. The massive size of the neural tube compared to the fetus body and its wide differentiation require optimal levels of oxygen and maternal mediators, such as folate, cobalamin (vitamin B12), thyroid hormones, serotonin, and melatonin. Brain development depends directly on the proper maternal-placental-fetal exchange.

Melatonin has a beneficial effect from the very early stages of pregnancy and the absence of pineal melatonin in the fetus makes the maternal and placental melatonin vital for the fetal brain development. Melatonin was detected in rat brain prior to the maturation of the pineal gland and during the neonatal period precedent to pineal activation. The origin of this melatonin remains to be determined. Central nervous system is a target to oxidative damage due to its high-energy consumption, low levels of endogenous antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD), high cellular content of lipid, and increased levels of cell differentiation and proliferation [106, 107]. The alteration of brain oxygenation leads to a wide variety of neuropathic lesions (intracranial hemorrhage and periventricular leukomalacia), mainly due to lack of neuronal myelination which increases glial cells instead of neuronal cells [108, 109]. Husson *et al*., have evaluated the impact of melatonin against neuronal white matter cysts mimicking human periventricular leukomalacia in murine model. Melatonin did not reduce the formation of neuronal lesions, but reduced by 82% the cysts and generated axonal growth and neuronal sprouting. These protective actions of melatonin appear to be mediated by its MT1 and MT2 receptors [110].

Other tryptophan derivatives molecules such as serotonin, the direct melatonin precursor, have a long-term effect on fetal brain development and might have long-term effects on mental health [36]. Serotonin has been recently related to the lack of myelination during brain formation, a situation that could be the origin of brain malformation and behavioral dysfunctions [111, 112]. Interestingly, Bonnin *et al*. has shown that it is the serotonin produced *de novo* by the placenta that is involved in the forebrain maturation in mice [36]. Nevertheless, role of placental melatonin and serotonin on fetal brain development remains to be studied.

From all modulators of fetal brain development, melatonin is one of the most susceptible to environmental variations (e.g. season of the year, luminosity, maternal nutrition, sleep-awake cycle, geographic location…) [23, 113]. The modulation of maternal melatonin production can have long-term consequences and even if experienced prior to a pregnancy, it can still affect (positively or negatively) fetal development [114]. It has been suggested that inheritance is possible via melatonin modulation of epigenetic profiles during the fetal development [77]. Melatonin plays a role in epigenetic modulation throughout the activation of the nuclear receptors (ROR and RZR families) leading to the modulation of DNA environment, or a direct interaction with the DNA methyl transferase (DNMT). Melatonin prevents the interaction of DNMT with the DNA (i.e. block DNA methylation). The small size and the amphiphilic profile of melatonin are characteristics that reinforce the latter hypothesis. As epigenetic inheritance system is considered an environmental mechanism for the DNA management, the susceptibility of melatonin to environment variations could be an impor

tant pathway by which it influences ectoderm cells, which differentiate into the neural tube in the very beginning of pregnancy [4, 115, 116].

Folate and Melatonin Synergetic Effect on Neural Tube Development

Maternal folate supplementation has been shown in epidemiological studies as a robust method to reduce the occurrence and even the recurrence of neural tube defects. Neural tube defects are the major cause of birth defects and are positively correlated with lower folate absorption, transport and cellular internalization [117, 118]. Folate poorly diffuses throughout the cell membranes and requires specific carriers, the reduced folate carrier proteins. These carrier proteins are highly expressed in human trophoblasts [119, 120]. A synergetic protective effect of folate and melatonin on brain development in the first stages of pregnancy has been recently shown in mice [121]. Lipopolysaccharide (LPS) is used to induce inflammation and has been shown to disrupt placental folate transport, thereby generates neural tube defects. This disruption of folate transport is reversed by melatonin treatment leading to a significant improvement of the neural tube formation and differentiation of brain areas. Maternal exposure to low levels of LPS during increased inflammatory status down-regulates the placental folate carrier proteins and prevents the transfer of folate to the fetal mice [122]. The activation of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 is intimately related with increased activation of endoplasmic reticulum (ER)-stress. ER-stress activation inhibits the proper formation of proteins, including folate carriers. Melatonin is a powerful anti-inflammatory agent able to decrease TNF-α expression and also acts as an ER-stress inhibitor [123, 124]. However, if melatonin can protect folate carrier disruption throughout an anti-inflammatory action and/or ER-stress inhibition remains to be studied.

Fournier *et al.* have shown that reduced level of folate alters melatonin synthesis and thus decreases melatonin serum levels. In parallel to the adverse effects on melatonin system, reduced levels of folate leads to homocysteine accumulation and disruption of oxidative balance [125]. Homocysteine is also a modulator of neural tube formation. Accumulation of homocysteine is positively correlated with malformations of the neural tube and increased neuronal apoptosis [126]. The molecular mechanisms involved in the fetal toxicity caused by a hyper-homocysteinemia however remain unknown. Some have hypothesized that higher levels of homocysteine could lead to excessive homocysteinylation of proteins [127]. Homocysteine is an amino acid negatively correlated with the metabolism of folate and cobalamin (also known as vitamin B12). Interestingly, it has been shown that melatonin can reduce the oxidative stress caused by homocysteine accumulation [128, 129]. The ability of melatonin to regulate the concentration of folate, homocysteine and cobalamin, crucial for fetal brain formation, indicate that melatonin is vital for proper brain formation *in utero*.

PROTECTIVE EFFECT OF MELATONIN ON PRETERM-BIRTH BRAIN DEVELOPMENT

Preterm birth involves delivery prior to 37 weeks of pregnancy and is associated with high perinatal morbimortality. Maternal infections, inflammation, stress, multiparity, preeclampsia, gestational diabetes mellitus, and IUGR are the main causes of preterm birth. The majority of preterm birth are idiopathic and probably associated with lifestyle and genetic polymorphisms [130]. Preterm birth is the main cause of cerebral palsy, intellectual disabilities and mortality at less than 5 years old [131, 132]. Preterm newborns have a delay of twenty days of excretion of the metabolite 6-sulfatoxymelatonin, urinary marker of plasmatic melatonin, compared with term babies. This suggests an immature pineal gland, the source of plasmatic melatonin in preterm babies [133]. Carloni *et al.* showed in rats that the lack of oxygenation could damage neuronal tissues with short and long-term consequences even after the parturition. Melatonin treatment before and after ischemia-reperfusion plays protective effects in a dose-dependent manner, as evidenced by behavioral tests. Also, melatonin acts more evidently on hippocampus, the brain structure that is more susceptible to oxidants effects [7, 134].

Brain during development, is highly susceptible to any disturbance. Melatonin has been shown to be safe, despite of the treatment period and the concentrations tested. In animal models, intraperitonial LPS induces preterm birth. In a recent study, pregnant mice were challenged with LPS in parallel with continuous melatonin treatment. LPS challenge generated 100% of preterm births with high rates of offspring morbimortality. Melatonin treatment was able to reduce by 50% the cases of preterm birth and totally abolished fetal mortality. Melatonin also reduced, to basal levels, inflammatory factors increased by LPS in this animal model, confirming the protective effect of melatonin [135]. The antioxidant effect of melatonin was also evaluated in animal models of preterm birth. Besides its anti-inflammatory effects, this indolamine is also able to avoid the aberrant oxidative stress levels generated by ischemia-reperfusion in preterm newborn brain of rodents and ovine experimental models. Melatonin exerts this protective effect via the upregulation of SOD and glutathione peroxidase and inhibition of lipid peroxidation and protein carbonylation. Melatonin also prevents neuronal apoptosis thru the maintenance of mitochondria integrity. As demonstrated by Watanabe *et al*., and Hutton *et al*., optimal protection was obtained with prolonged treatments throughout the pregnancy even with low doses (0.1 mg/kg/day) [136-140].

To date, no major adverse effect has been described in children and adults after chronic or acute treatments with melatonin [138, 141-143]. Furthermore, exogenous melatonin does not suppress endogenous melatonin secretion; currently it is used as an adjuvant for the control of the circadian cycle [144]. Melatonin clinical trials to protect preterm newborns are currently conducted. As reviewed by Biran *et al.,* the aim of melatonin clinical trials conducted with preterm newborns is to evaluate the doses, the length of treatments, and the pharmacokinetics profile [8]. A phase two double-blinded clinical trial (ISRCTN01115788, 2010, NCT00649961, 2014) evaluated the effect of melatonin on preterm neonates. The main objective was to determine if brain lesions could be reduced. Another clinical trial currently conducted aims to access the effect of melatonin treatment to reduce the impact of preterm birth on the neonate brain (NCT02395783). This study evaluates the optimal maternal dose to decrease neonate neuronal damages. A study has demonstrated that exogenous melatonin, in association with hypothermia, increased melatonin plasmatic levels in preterm neonates, which was able to increase the neuronal viability leading to the absence of motor and behavioral abnormalities [145]. On the other hand, another study did not find any improvement in preterm newborns treated with melatonin daily for one week in comparison with the placebo (saline) group [146]. The disparity of these recent results shows that melatonin is a potential treatment for injuries caused by the lack of oxygenation due to preterm birth or other pregnancy diseases. However, the length of the treatment, the concentration chosen and even co-treatments are aspects that should be evaluated to improve its protection.

CONCLUSION

Placental insufficiency caused by inadequate oxygenation, as observed in pregnancy disorders, generates important effects on fetal development and brain formation [147-149]. Throughout the gestation, fetal demand of oxygen is progressively increased due to metabolic consumption, which is dependent of vasculogenesis during the first trimester. Also during the first trimester neurogenesis is already activated and is strongly dependent to placental oxygena-

tion and maternal environmental factors, such as lifestyle and nutrition. The absence of endogenous antioxidant defenses turns fetal brain highly sensitive to any disturbance; hence fetal brain is susceptible to pregnancy disorders. Melatonin via its receptors and antioxidant effects has been shown as a possible treatment in these situations [54, 150]. Indeed, some authors suggest that in parallel to its classic actions, melatonin could have long-term effect (i.e. several generations) by the modulation of the superstructure of DNA. This latter action could be related with activation of specific inheritance. In sum, melatonin is able to modulate the production of pregnancy hormones and improve placental cell viability; in parallel, it could upregulate the brain development and programing during the fetal period with long lasting consequences.

LIST OF ABBREVIATIONS

AMK	=	N1-acetyl-5-methoxykynuramine
CAT	=	catalase
COX	=	cyclooxygenase
DNMT	=	DNA methyl transferase
ER	=	endoplasmic reticulum
GPCR	=	G protein coupled receptors
iNOS	=	inducible nitric oxide synthase
IL	=	interleukin
IUGR	=	intrauterine growth restriction
LPS	=	lipopolysaccharide
Nrf2	=	nuclear factor erythroid 2-related factor 2
ROR	=	retinoid orphan receptor
RZR	=	retinoid Z receptor
SCN	=	suprachiasmatic nucleus
SOD	=	superoxide dismutase
TNF-α	=	tumor necrosis factor alpha

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ANNEXE 3 – ARTICLE DE REVISION : MATERNAL AND PLACENTAL MELATONIN: ACTIONS AND IMPLICATION FOR SUCCESSFUL PREGNANCIES.

Contribution de l'étudiant

Lucas Sagrillo-Fagundes a participé à la validation des sujets de l'article. Il a également rédigé l'article et effectué les corrections demandées par les évaluateurs.

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Maternal and placental melatonin: actions and implication for successful pregnancies

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Melatonin is one of the main sources of mitochondrial protection and its protective effects are equal or even better if compared with several consecrated antioxidants. Furthermore, the activation of specific melatonin receptors triggers several cellular pathways that improve the oxidoreduction and inflammatory cellular state. The discovery of the melatoninergic machinery in placental cells was the first step to understand the effects of this indoleamine during pregnancy. In critical points of pregnancy, melatonin has been pointed as a protagonist and its beneficial effects have been shown as essential for the control of trophoblastic function and development. On the contrary of the plasmatic melatonin (produced in pineal gland), placental melatonin does not vary according to the circadian cycle and acts as an autocrine, paracrine, intracrine, and endocrine hormone. The important effects of melatonin in placenta have been demonstrated in the physiopathology of pre-eclampsia with alterations in the levels of melatonin and in the expression of its receptors and synthetizing enzymes. Some authors suggested melatonin as a biomarker of pre-eclampsia and as a possible treatment for this disease and other obstetric pathologies associated with placental defect and increases in oxidative stress. This review will approach the beneficial effects of melatonin on placenta homeostasis and consequently on pregnancy and fetal health.

KEY WORDS: Melatonin - Placenta - Pre-eclampsia - Pregnancy.

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Melatonin (*N*-acetyl-5-methoxytryptamine) is a molecule with wide protective effects that was thought to be exclusively produced in the pineal gland of superior animals.[1] However, it is well known now that its synthesis is virtually present in every cell of every organism, from bacteria throughout the animal and plant kingdoms.[2] This indoleamine is synthetized from the L-tryptophan pathway in a multistep process that has serotonin as an intermediate precursor. The serotonin is acetylated by the enzyme arylalkylamine *N*-acetyltransferase (AANAT) in *N*-acetylserotonin that is finally converted in melatonin by the enzyme serotonin *N*-acetyltransferase (ASMT, also known as hydroxyindole O-methyltransferase [HIOMT]). High concentrations of melatonin and the presence and activity of AANAT, considered the rate-limiting enzyme in melatonin synthesis production, in the mitochondrion assure that melatonin is produced at the cytoplasm and in the mitochondria.[3] Tan *et al.* (2013) suggested that this indoleamine was originally produced in the intermembrane space of the mitochondria and has migrated to the cytoplasm throughout the eukaryo-

tic evolution.[4] As mitochondria are the main cellular source of reactive species and consequently of oxidative stress, the massive presence of melatonin demonstrates its powerful antioxidant protection and is associated with the maintenance of the homeostasis in this organelle.[5] Besides the endogenous production of melatonin, it can also be obtained from exogenous sources. As melatonin is highly preserved in several species, it can be absorbed by the consumption of a variety of nuts, fruits, and vegetables.[6]

Melatonin produced by the pineal gland is delivered in the blood and cerebrospinal fluid and is responsible for the control of the circadian and circannual rhythms.[3] This photoneuroendocrine role of melatonin has been extensively studied and is related to variations on the reproductive competence. Equivalently to the universal phylogenic distribution of melatonin, its presence and production are attributed to several cellular types and organs, however only the pineal melatonin is available in the plasma; the synthesis in other organs is locally used with an autocrine and paracrine profile.[5] In other fluids than the blood and locally in organs where the production is well established, like intestine, skin and placenta, in general the concentration of melatonin exceeds the plasmatic levels. Conversely to the pineal production of melatonin, the production in these organs and fluids is not influenced by the variation of the diurnal length and photoperiod seasons.[7, 8] Besides the influence of melatonin to regulate the physiological alterations caused by the length of night's variations, it is also related to protective roles as free radicals scavenging, anti-inflammatory actions, improvement of immune system and an anti-cancer activity mediated by several cellular pathways.[2, 9]

Melatonin is highly soluble in lipophilic solutions and partially soluble in water, and this amphiphilic profile allows the prompt crossing of all physiological and cellular barriers. This characteristic allied to its intermediate size allows the presence of melatonin in all cells and its compartments.[9] The cytoprotective effect of melatonin goes beyond its direct antioxidant effect and is also related to the actions mediated by its receptors. The melatonin receptors type 1 and type 2 (MT1 and MT2, respectively) are members of the G protein-coupled receptors family (also known as seven-transmembrane domain receptors) and possess 60 % of homology in their sequence of amino acids.[3, 10] The effect of the interaction between melatonin and its receptors is variable regarding the tissue, the type of cell and the cellular phase. When MT1 and MT2 are activated, different isoforms of protein G lead to the activation of different signalling pathways (Table I).[3, 11-15] Melatonin is also able to interact with several members of the ROR/RZR (retinoid orphan receptor/retinoid Z receptor) nuclear receptors family. Regardless their affinity to melatonin, the controversial information that melatonin is their native ligand is still deliberated.[3] MT1 and MT2 are widely spread and are present in virtually all cells of the human body. MT1 is the main responsible of the regulation of the circadian cycle and is principally found in the central nervous system, whereas MT2 is related to the control of the body tem-

TABLE I.—*Cellular pathways modulated by melatonin and its receptors.*

Pathway	Effect of melatonin	Details	
Ca^{2+} channels	Inhibition	Inhibits the complex Ca^{2+}/calmodulin and stimulates the cytoskeleton polymerisation	3
K^{+} channels	Activation	Activated *via* PKA pathway	11
PLC	Activation	Activates ERK1/2	12
cAMP/PKA	Inhibition	Pathway inhibited via G_i	13
ERK1/2	Activation	Activated under oxidative stress	14
PLA2	Activation	Activated under oxidative stress	15

cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; PLC: phospholipase C; ERK1/2: extracellular-signal-regulated kinases; PLA2: phospholipase A2.

perature and is spread by the peripheral tissues.[3, 5] Several effects attributed to melatonin are related to its receptors, such as the stimulation of the expression of the antioxidant enzymes, regulation of the arterial tension and the inhibition of the expression of inflammatory factors.[16-18]

The increased interest and research on the cytoprotective action of melatonin are attributed to its unique characteristics. In a recent review, Galano *et al.* (2011) elegantly summarized the beneficial characteristics that qualify melatonin as an ideal radical scavenger molecule.[9] Melatonin has been proven to be versatile and is capable to mitigate several oxidants by mechanisms such as: Single electron transfer (SET: $Melatonin + {}^{\bullet}R \rightarrow Melatonin^{\bullet+} + R^{-}$) and Radical adduct formation (RAF; $Melatonin + {}^{\bullet}R \rightarrow Melatonin\text{-}R^{\bullet}$). SET has been proposed as the main antioxidant mechanism of indole derivatives (*e.g.* tryptophan, melatonin, serotonin) against different oxidants.[19] This mechanism is characterized by the donation of an electron from melatonin to the radical and the consequent reduction of the reactivity of the latter and the formation of a less reactive radical from melatonin (expressed in the equation as Melatonin$^{\bullet+}$). The hydroxyl radical ($^{\bullet}$OH) is the most devastating free radical. It is normally produced intracellularly, but can also be generated by ultraviolet and ionizing radiation. The antioxidant activity of melatonin is effective against this radical by the RAF mechanism. Different techniques demonstrated that $^{\bullet}$OH reacts and is inactivated by the association with the carbon sites of melatonin. In this specific situation, the broad distribution of melatonin in different organelles, cells and body compartments is primordial to cut down the effect of $^{\bullet}$OH.[20, 21] The inactivation of $^{\bullet}$OH by melatonin generates a metabolite, cyclic-3-hydroxymelatonin (3-OHM), which has a weak reactivity and no cellular toxicity (in the RAF equation above, R$^{\bullet}$ represents $^{\bullet}$OH and 3-OHM is expressed as $Melatonin\text{-}R^{\bullet}$). The detection of this compound in the urine is a marker of the protection of melatonin against the $^{\bullet}$OH radical.[21] This metabolite of melatonin also acts as antioxidant, as well as other metabolites formed from the reaction between melatonin and reactive species, like N^2-acetyl N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK) (Figure 1).

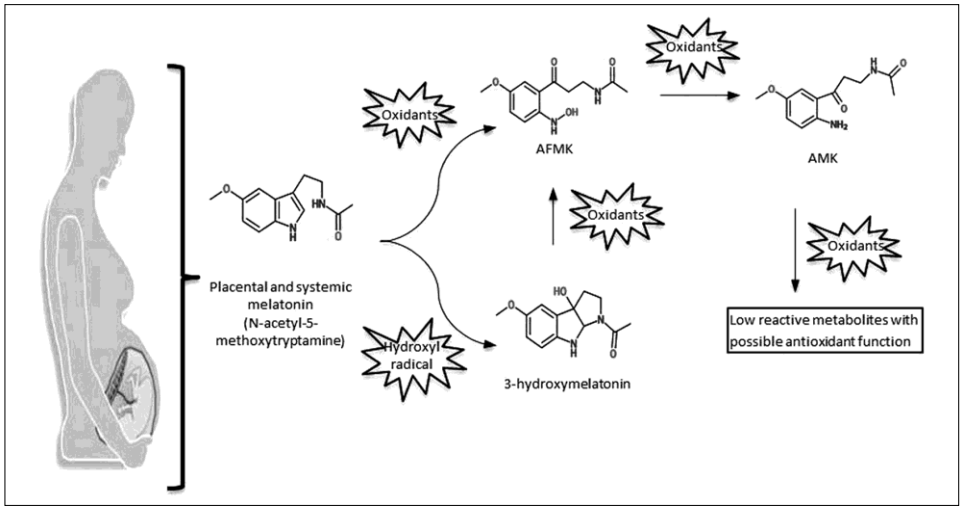

Figure 1.—Melatonin antioxidant cascade. Radical scavenging activity of melatonin that is found in several organs, among them the placenta. The melatonin metabolites have been shown as important antioxidant agents. AFMK: N^2-acetyl N^2-formyl-5-methoxykynuramine, AMK: N^1-acetyl-5-methoxykynuramine.

The importance of the metabolites of melatonin as antioxidants is already well established and there are evidences that AMK is even more potent than melatonin under specific situations.[9, 22] This unique feature distinguishes melatonin from several antioxidants and makes melatonin highly effective, even in low levels.[23]

Physiologically, the aerobic respiration generates reactive species of oxygen and nitrogen with an oxidant profile, called reactive oxygen species (ROS) and reactive nitrogen species (RNS). An unbalance between the levels of reactive species and antioxidants with an advantage for the formers is called oxidative stress. This situation is caused by an important reduction of antioxidants, increase of oxidants and the combination of both factors.[24] Mitochondria are the main cellular source of ROS and RNS, more specifically the complexes I and III of the electron transport chain (ETC). Even under normal conditions, 1-2% of the electrons are not completely reduced leading to the production of the anion radical superoxide ($O_2^{\bullet -}$). Subsequent reactions create several products, also with a reactive profile, such as hydrogen peroxide (H_2O_2), oxygen singlet ($^1O_2$) and the nitrous acid (HNO_2).[22, 24] Melatonin is able to improve the ETC efficiency, with a consequent reduction of free radicals generation. The wide localisation of melatonin, associated with its prolonged antioxidant activity and the stimulation of antioxidants enzymes expression are also sufficient to inactivate the free radicals effects and reduce the oxidative stress.[25]

Autophagy is the main cellular mechanism to eliminate cellular components (including mitochondria) that are dysfunctional or were affected by the oxidative stress with the aim to avoid the cellular death. This mechanism is constantly active and is one of the responsible to maintain the placental homeostasis, in spite of the constant alterations and stressors that affect this organ. The modulation of melatonin on the antioxidant enzymes of mitochondria and the direct effect of this indolamine as an oxidant scavenger demonstrate its mitochondrial protective effect. Recently, the relationship between the treatment with melatonin and the improvement of the homeostasis by the increase of autophagy and mitophagy (autophagy of mitochondria) was defined. However, Coto-Montes *et al.* demonstrated that, on time, there are no evidences to determine if the effect of melatonin on autophagy is related to its antioxidants effect or related to the action *via* its receptors.[26, 27]

The effect of melatonin on the inflammatory pathways is characterised by endocrine, autocrine, intracrine and paracrine responses. Markus *et al.* recently developed a hypothesis of the mutual control of melatonin and the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). Melatonin is considered a key factor of the immune system control and NF-κB can inhibit (in pinealocytes) or induce (in macrophages) the production of melatonin by the control of the enzyme AANAT.[18] Equivalently, tumor necrosis factor (TNF) also inhibits the production of melatonin by the suppression of AANAT and is inhibited under the treatment of melatonin.[28] Melatonin can inhibit, *via* its receptors and *via* free radical scavenging, the expression and activity of inflammatory mediators involved in the pathogenesis of several diseases of pregnancy, such as inducible nitric oxide synthase (iNOS), interleukin-2 (IL-2) and cyclooxigenase-2 (COX-2).[29, 30]

The "pleiotropic" effects of melatonin are extremely wide and include 1) mitochondrial homeostasis regulation; 2) induction of antioxidant enzymes; 3) inactivation of ROS and RNS; and 4) regulation of autophagy and inflammation. These actions are responsible for the cellular regulation and are extremely important for the cellular protection and also against challenging situations, commonly found during pregnancy.

Melatonin and pregnancy

Since the conception, the protective effects of melatonin are related to the improvement of the fertility and across the

pregnancy there is a continuous raise of plasmatic melatonin levels with the maximum during the parturition. During pregnancy, melatonin levels are higher during the nights than those measured in non-pregnant women. Interestingly, if compared to the non-pregnant women (whose melatonin is almost undetectable during the day), the diurnal levels of melatonin are high during pregnancy.[31] This variation of melatonin is hypothesised to be related with melatonin production by placental cells, which are also targets of this indoleamine. Despite of the site of melatonin production, it is essential for fetal development. The maternal variation of melatonin during pregnancy is also measured in fetal circulation and situations of circadian cycle alterations affect the expression of rhythmic genes in the fetus, which is reversed with daily melatonin injections.[32, 33]

Implantation

Local production of melatonin by human ovary was first established upon detecting its functional synthesizing enzymes (AANAT and ASMT) by Itoh *et al.*[34] Then the expression of melatonin receptors (MT1 and MT2) was revealed, being localized in human granulosa and luteal cells.[35] These findings explain – in part – the high melatonin level detected in ovarian follicular fluid compared to blood samples [7] giving rise to numerous research projects concerned with exploring the role of melatonin in ovarian functions.

The ovulation process is associated with inflammatory reactions.[36] Recruitment of macrophages, leucocytes and vascular endothelial cells in ovarian follicle results in elevated levels of inflammatory mediators and ROS.[37] Although ROS play a role in ovarian follicle rupture, elevated levels could result in diminished progesterone production by luteal cells and low oocyte quality due to oxidative injury.[38] Since melatonin is well-known to exert a powerful antioxidant effect through receptor-dependent and independent actions, high melatonin levels observed in preovulatory follicles compared to immature ones confirm its essential role in successful ovulation with high oocyte quality.[39]

In a clinical study with 25 infertile patients diagnosed with luteal phase defects (low serum progesterone level less than 10 ng/mL), melatonin administration to 14 patients (3 mg/day/oral) through luteal phase improved progesterone concentration in nine of them. On the other hand, the 11 non-treated patients experienced a non-significant increase in progesterone levels post-hCG injection. These results confirm ROS association with luteal phase defects and underline the antioxidant role of melatonin in protecting granulosa cells while enhancing progesterone production by the corpus luteum.[40]

Although assisted reproductive technologies (IVF-ET) have been significantly improved, poor oocyte quality remains the major challenge due to oxidative destruction.[41] Intriguingly, melatonin's powerful antioxidant effects through free radical scavenging, and increased antioxidant enzyme expression, augmented oocyte quality with extraordinary IVF-ET success rates.[17, 42] In 18 patients undertaking IVF-ET, melatonin administration (3 mg/tablet/day) at night from the 5th day of the previous menstrual cycle until the day of oocyte retrieval, triggered a higher intrafollicular melatonin level compared to controls. Remarkably, melatonin-treated patients experienced reduced intrafollicular concentrations of 8-hydroxy-2-deoxyguanosine (8-OHdG) and hexanoyl-lysine adduct (HEL), biomarkers of DNA damage and lipid damage, respectively.[43, 44]

To examine melatonin's usefulness on IVF-ET clinical outcome, 115 patients who failed to achieve pregnancy during the previous IVF-ET cycle and had a low fertilization rate (less than 50%) were recruited (Table II). Compared to previous cycle results, melatonin administration (3 mg/day) to 56 patients improved the fertilization rate with 11 patients achieving pregnancy. Conversely, for the 59 non-treated patients, no substantial change was observed in the fertilization rate and only six got pregnant.[43, 45]

TABLE II.—*Melatonin administration enhances in vitro fertilization pre-embryo transfer (IVF-ET) clinical outcome.*

	Melatonin administration 56 cycles	No melatonin administration 59 cycles
Fertilization rate in previous IVF-ET cycle	20.2±19%	20.9±16.5%
Fertilization rate	50±38%*	22.8±19%
Pregnanacy rate	11/56 (19.6%)	6/59 (10.2%)

* P<0.01 compared with the previous IVF-ET cycle (Mann-Whitney U-test)
Data from Tamura *et al.*[43]

These findings unravel a role for intrafollicular melatonin in reducing oxidative stress, protecting oocytes from injury and improving IVF-ET pregnancy success rates. Also demonstrate the effectiveness of oral melatonin supplementation during IVF-ET procedures, achieving enhanced oocyte quality in non-fertile women.

The aforementioned studies on oral melatonin administration and successful IVF-ET pregnancies support Tamura *et al.*[45] suggestion of plasmatic melatonin entry into ovarian follicular fluid. Subsequently, this suggests that *de novo* synthesized ovarian melatonin along with plasmatic melatonin might contribute to the high melatonin level observed in follicular fluid. One could propose that the activities of maternal melatonin could be associated with the reported high melatonin levels, which contribute to the high oocyte quality.

Parturition

Parturition or childbirth is the time of pregnancy culmination that involves spontaneous and vigorous myometrium contractions, resulting in cervical effacement and fetal descent. Published reports revealed parturition as a light sensitive event with an onset likely to occur at late night and early morning hours.[46] Although full explanation of this phenomenon is still ambiguous, some researchers expected that childbirth during darkness would guard both the mother and fetus from predators and maximize their survival, since labor process is known to be associated with maternal exhaustion and weakness.[47]

Irrespective of the role of oxytocin in parturition and its use for labor induction, its levels showed minimal increase during labor. Moreover, typical parturition occurred in the absence of posterior pituitary gland influence, after its removal.[48] Since parturition follows circadian rhythm as a nocturnal event, pineal gland melatonin was suspected to share a regulatory effect on this process. In this line of thought, Takayama *et al.*[49] examined the effect of melatonin injection on parturition timing in pinealectomized rats. This study revealed melatonin's importance in restoring normal parturition peak and maintaining photoperiodic rhythm synchronization. Similarly, a recent clinical study on pregnant women volunteers confirmed the role of night-time melatonin in augmenting uterine contraction. These women (at their >38 gestational week) were continually inspected for their uterine contractions from 7:00 pm till 7:00 am under dim light, and then they were eye-exposed to full spectrum light (10,000 lux) at 11:00 pm for 1 hour. Since this light spectrum is known to suppress nocturnal melatonin secretion,[50] uterine muscle contractions were shown to be impeded, which started following light exposure for the remainder of the night.[14] Owing to the widespread misuse of artificial light at night, this could explain the present-day drop in night-time melatonin along with nocturnal delivery.

Aside from the fact that uterine myometrium expresses both oxytocin and melatonin receptors,[51] melatonin and oxytocin activate the same signaling pathway of phospholipase C (PLC), protein kinase C (PKC) and myosin light chain kinase leading to uterine muscle contraction and offspring expulsion (Figure 2).[52, 53] While myometrial cells treated with low concentrations of oxytocin failed to show full contractions *in vitro,*

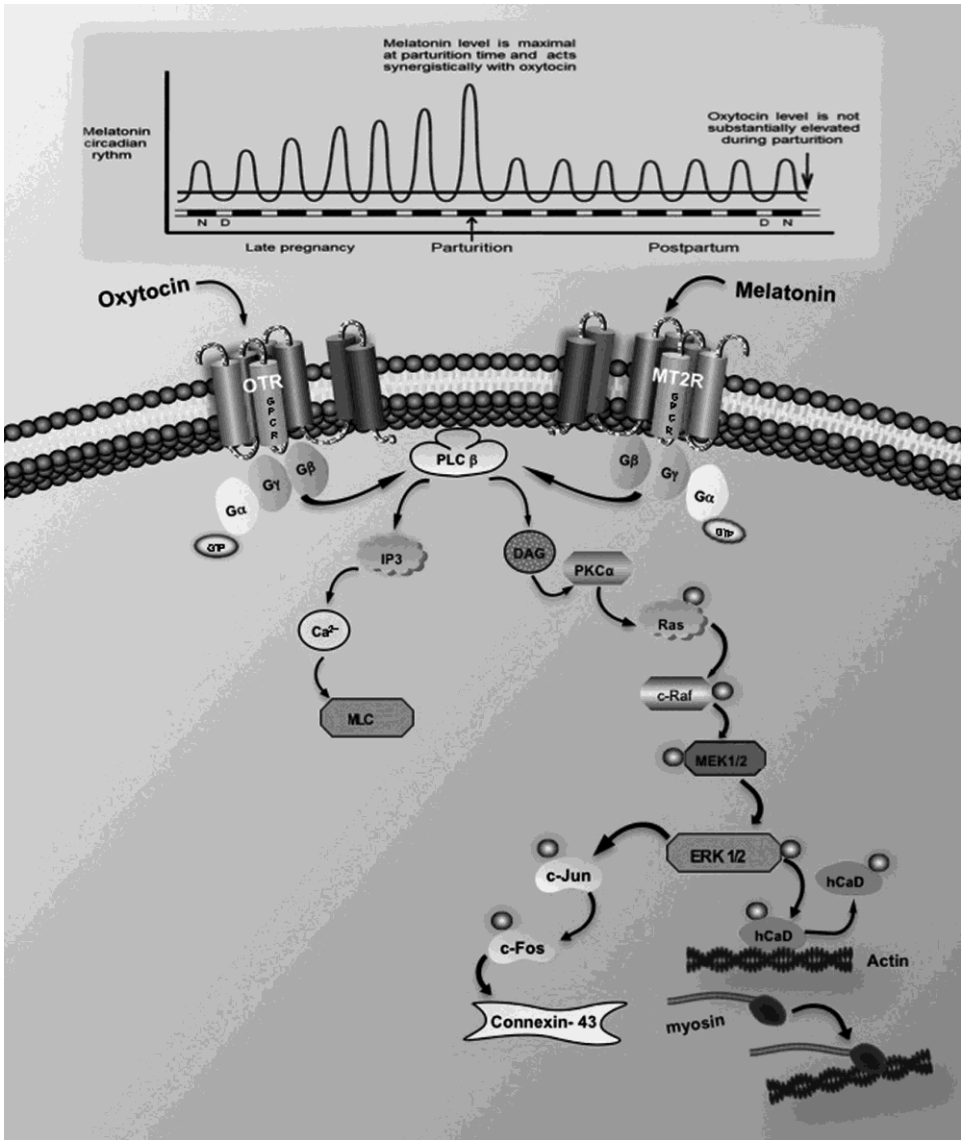

Figure 2.—Suggested model of synergetic action of melatonin and oxytocin on night-time myometrial contractility at parturition time. Melatonin circadian rhythm and oxytocin level is described through the last trimester of pregnancy, at parturition time and after delivery of human females. Melatonin and oxytocin stimulate respectively melatonin type 2 (MT2R) receptor and oxytocin (OTR) receptor to synergistically activate PLCβ, which hydrolyses PIP2 into DAG and IP3. DAG activates PKCα, activating the Ras/Raf/MAPK signaling pathway and leading to hCaD phosphorylation. This increases actin availability for myosin binding and contractility induction. Moreover, PLCβ stimulates both c-Jun and c-Fos, resulting in enhanced gap junctional communication *via* connexin 43 activation. Finally, IP3 stimulates $Ca2^{+}$ release that activates MLC and enhances myometrial contractility. PLC: phospholipase, PIP2: phosphatidylinositol 4, 5-bisphosphate, DAG: diacylglycerol, IP3: inositol 1, 4, 5- triphosphate, PKC: protein kinases type C, MAPK: mitogen-activated protein kinase, hCaD: caldesmon, MLC: myosin light-chain. Adapted from Reiter *et al.*[52]

melatonin supplementation (1 nmol/L) acted synergistically and sensitized uterine muscle to oxytocin resulting in their peak contraction. A possible explanation for the synergistic action of melatonin is the up-regulation of the activity and expression of connexin 43, a gap junction protein, consequently improving intracellular communication and myometrial contraction synchronization. Furthermore, laboring pregnant women showed corresponding upregulation of melatonin and oxytocin receptors compared to pregnant non-laboring ones.[14]

Interestingly, night-time circulating melatonin increases in late pregnancy reaching its peak by delivery time,[54] as shown by its levels in both maternal amniotic fluid [55] and urine [56] near term. Also nocturnal melatonin levels drops to normal (non-pregnant women level) instantly after delivery,[31, 57] assuming melatonin significance in determining parturition time through tracking light/dark cycle. Since placental melatonin levels have been shown to contribute to increased maternal blood levels of melatonin (*discussed in details in Melatonin and placenta section*) we could propose that it has a role in the parturition process due to its leakage into maternal blood or due to its paracrine activities on myometrial melatonin receptors.

In summary, the mentioned findings could explain the higher melatonin levels observed in late pregnancy as well as elevated night time delivery. It would be valuable to recommend melatonin supplementation at the onset of spontaneous labor, to surge uterine contractions and lessen its period as well as relieving delivery aches.[58]

Amnion and amniotic fluid

Amniotic sac is a transparent, thin and strong dual membrane bag inside which the fetus grows, the inner membrane is called amnion and includes both the amniotic fluid and the developing fetus, while the outer membrane is called chorion and forms part of the placenta. Together with the placenta and umbilical cord, amniotic sac is pushed out of the mother upon delivery.[59] In 1978, Mitchell *et al.*[60] detected high melatonin concentrations in the amniotic fluid during labor even greater than those observed in late pregnancy, questioning if melatonin might exert functional role during parturition. Interestingly, those findings match present-day data explaining melatonin activities during parturition accompanied by its high nocturnal level in late pregnancy, peaking by labor time.[61]

As melatonin is transferred easily and rapidly from maternal to fetal circulation,[62] Xu *et al.*[63] reported that maternal pretreatment with melatonin attenuated the lipopolysaccharides (LPS)-induced pro-inflammatory cytokine TNF presence in the amniotic fluid, emphasizing the anti-inflammatory action of melatonin. Moreover, melatonin receptor MT1 is expressed in the amniotic epithelial cells, which through melatonin stimulation increased cell proliferation. Interestingly, melatonin pre-treatment reversed amniotic epithelial cells demise after H_2O_2 exposure and promoted their survival, thus illustrating melatonin antioxidant activities in the amniotic epithelial cells.[64]

Because of the limited number and controversial findings of present-day studies on the involvement of melatonin in the amniotic fluid,[53, 65] extensive research is required to determine if melatonin is produced locally by amniotic membrane cells or due to its outflow from the placenta and maternal blood, or both. Moreover, it will be important to determine if amniotic fluid melatonin and amniotic sac melatonin receptors have implications in both normal pregnancy and various pregnancy complications.

Melatonin and placenta

Human placenta is a transitory organ that attaches the developing fetus to the wall of maternal uterus, where it plays a chief role in pregnancy well-being and fetal growth.[66] Human placenta mediates feto-maternal exchanges of nutrients, ions and oxygen as well as elimination of fetal waste products and metabolites. Furthermore, it functions

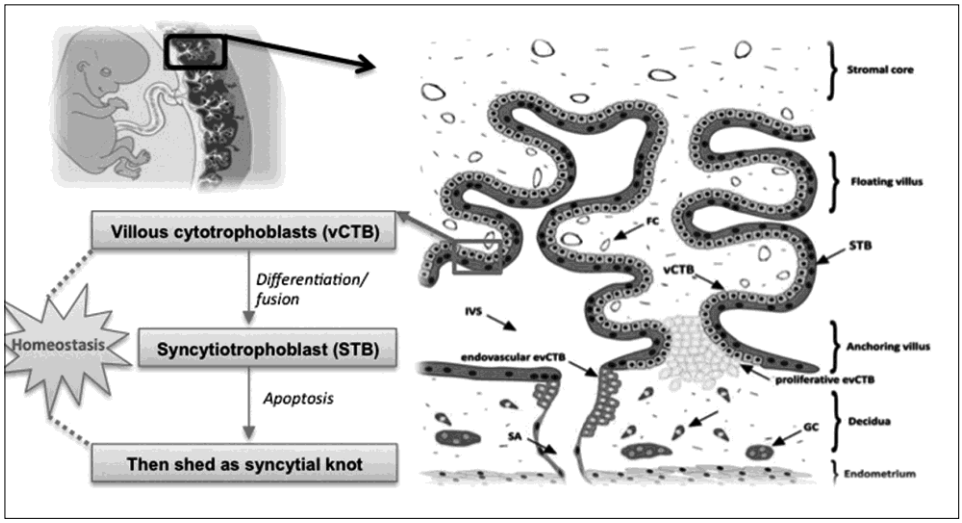

Figure 3.—Human villous trophoblast differentiation. Villous cytotrophoblast (mononucleated) aggregate and fuse into syncytiotrophoblast (multinucleated) in a highly organized process. SA: spiral artery; GC: giant cell; evCTB: extravillous cytotrophoblast; FC: fetal capillary STB: syncytiotrphoblast; vCTB: villous cytotrophoblast; IVS: intervillous space. From Zeldovich *et al.*[68] and Vaillancourt *et al.*[71]

as an endocrine organ producing specific steroid and peptide hormones, while protecting the fetus from being rejected by the maternal immune system through its immunomodulatory activities.[67]

The functional unit of the human placenta is the chorionic villi (Figure 3), formed of mononucleated villous cytotrophoblasts, which aggregate and fuse into multinucleated syncytiotrophoblast in a highly controlled process. Assuring the villous trophoblast homeostasis is vital to pregnancy success and fetal development (Figure 3). During the course of pregnancy, the syncytiotrophoblast undergo apoptosis to allow its continual regeneration by the underlying proliferative villous cytotrophoblasts.[68] Factors that disturb villous trophoblast homeostasis such as oxidative stress increase syncytiotrophoblast loss by apoptosis and trigger pregnancy complications such as pre-eclampsia and fetal growth restriction.[69-71]

In 2005, Iwasaki *et al.*[72] anticipated melatonin regulatory activities in human placenta upon identifying both melatonin synthesizing enzymes (AANAT and ASMT) and melatonin membrane receptors (MT1 and MT2) mRNA transcripts in first trimester placental tissues. Afterwards, our team conducted further extensive studies on placental melatonin system, where we demonstrated mRNA and protein expression of melatonin receptors (MT1, MT2 and RORα1) in normal human term placenta and in human placental choriocarcinoma cell lines (JEG-3 and BeWo).[8, 73] Subsequently, through immunohistochemistry, RT-PCR, Western Blot and LC-MS/MS techniques, we localized functional AANAT and ASMT as well as the melatonin receptors (MT1, MT2 and RORα1) on both villous cytotrophoblast and syncytiotrophoblast of normal human term placenta.[8] Our findings were consistent with the observations of elevated extra-pineal melatonin production during pregnancy, as detected in maternal blood, which peaks by term and does not follow the circadian rhythm. This also matches propositions about the placental origin of melatonin during pregnancy. Since the level of melatonin returns to normal after delivery and is higher in twin pregnancies compared to single pregnancies,[72] this supports the idea that placental melatonin contrib-

utes to the high maternal blood level during pregnancy. As demonstrated by Nakazawa *et al.*, the melatonin content in human and rat placental tissue is high even during day hours. Even more, melatonin content in human placental tissues is 6 to 7-fold higher than in the plasma.[74]

A recent study by our team showed a protective effect of melatonin on villous cytotrophoblasts and syncytiotrophoblast in a situation that mimics the pre-eclampsia and generates high levels of oxidative stress, the exposition to an environment of hypoxia/reoxygenation (H/R).[75] Exposition of primary syncytiotrophoblast cell to H/R induced the activation of the apoptosis by the mitochondrial pathway, which was completely reversed upon treatment with 1mM of melatonin. In this model, melatonin treatment significantly increases the levels and activity of antioxidant enzymes (superoxide dismutase and glutathione peroxidase), reduces both pro-apoptotic factors (caspases 9 and 3) and the proteins of Bcl2 with a consequent protection of the mitochondrial integrity. Under oxidative stress, the raise of the permeabilization of the mitochondrial membrane releases activating factors of the intrinsic apoptotic pathway. This mechanism is regulated by members of B-Cell Lymphoma 2 (Bcl2) family, which are responsible for the formation of pores in the outer membrane of mitochondria and the liberation of pro-apoptotic factors (*e.g.*, cytochrome-c). These factors activate the apoptotic pathway with the cleavage of pro-caspase 9 and pro-caspase 3, and the consequent condensation of the chromatin and fragmentation of the DNA.[75, 76]

Modulation of antioxidants and oxidants levels and the consequent oxidative stress during pregnancy leads to a proper implantation and placental development.[69] For example, mild levels of oxidative stress regulate the syncytialization of the villous trophoblasts; however super-expression of the antioxidant enzyme superoxide dismutase impairs the switch from villous cytotrophoblasts to syncytiotrophoblast.[77] The increase of oxidative stress during pregnancy is related to the tension of oxygen, significantly increased in the uterine arteries in comparison with the intervillous space. This is essential for the placental exchanges and is a key-factor for the migration and invasion of the extravillous cytotrophoblasts and its differentiation to endovascular extravillous cytotrophoblasts. The optimal invasion of the extravillous cytotrophoblasts leads to the rise of the oxygen tension from <20 mmHg (first trimester) to 45-55 mmHg (second and third trimester), essential for the proper fetal development.[69] Improper extravillous cytotrophoblast invasion in the maternal decidua will alter the increase of oxygen tension, leading to complicated pregnancies, such as pre-eclampsia.[69]

Owing to our curiosity in identifying melatonin functions in placental physiology, we were the first to demonstrate that melatonin has a dual effect on apoptosis in tumor cells versus normal trophoblast cells. Melatonin induces apoptosis in BeWo choriocarcinoma cell line (tumor villous trophoblast) cells while inhibiting apoptosis and promoting survival in normal villous trophoblast. Melatonin prevents tumor cell proliferation through pro-apoptotic pathways activation and reactive oxygen species induction, *via* receptor dependent and independent activities, resulting in mitochondrial membrane permeabilization and cell death. By contrast, melatonin promotes non-tumor cell survival through its antioxidant properties and pro-apoptotic pathways inhibition, which occurs *via* a MT1/MT2 receptor pathway that inhibits Bax/Bcl-2 intrinsic apoptosis.[78] Nevertheless, further research is needed to unravel the mechanisms by which melatonin and its receptors preserve and regulate trophoblast homeostasis.

In sum, melatonin is *de novo* synthesized and its receptors are expressed in human placental trophoblastic cell, providing a unique model for the study of the mechanisms and signaling pathways involved in the protective actions of melatonin. Also, placental melatonin is strongly associated with the improvement of placental functions and development and with the protection

from oxidative stress, and is pointed as a potential watchdog against H/R-induced alterations in villous trophoblast homeostasis.

Pregnancy complications: melatonin and placenta

The comparison between the average weight of placentas and pineal glands indicates that both enzymes responsible for the production of melatonin, AANAT and ASMT, have their activity at least 2-fold higher in villous cytotrophoblast and in the syncytiotrophoblast compared to the pineal production. The concentrations of melatonin is 225-fold higher in placental cells.[8] These data show the massive level of melatonin in placenta, however its functionality remains partially understood. One role that has been characterized is its cytoprotective effect on trophoblast cells exposed to H/R.[75] During the first trimester of normal pregnancies, the invasion performed by extravillous trophoblasts is responsible for the improvement of the oxygen availability in the intervillous space, however, in preeclamptic placentas the lack of extravillous trophoblast invasion compromise the oxygen availability and the feto-placental homeostasis. Roberts and Hubel [79] distinguished pre-eclampsia in two phases. The first phase is defined as failure of vessel remodelling. The second phase is characterized by clinical signs and by the uncontrolled enhancement of placental oxidative stress, inflammation and erroneous expression of placental factors. These issues arise from the incomplete arterial remodelling leading to an insufficient or, more frequently, to intermittent blood supply, properly reproduced *in vitro* by a H/R exposure. As described previously, melatonin is effective to avoid the apoptosis and oxidative effects caused by H/R by the modulation of several steps of intrinsic apoptosis pathway and storage of oxidative status.[22, 77]

Aside the protection provided by melatonin to villous cytotrophoblast and syncytiotrophoblast in an environment that simulate pre-eclampsia, our group observed that preeclamptic pregnancies demonstrate decreased placental melatonin levels associated with lower expression of placental melatonin receptors, lower expression and activity of AANAT and lower activity of ASMT.[80] To define if the decrease of melatonin production was due to a lack of a precursor or an effect on its synthesizing enzymes, the level of serotonin was assessed and, on the contrary of melatonin, it was increased in pre-eclamptic placentas, which suggest the direct effect of pre-eclampsia on the melatoninergic synthesis machinery. This corroborates the results of Nakamura *et al.* (2001), which show decreased plasmatic melatonin levels in subjects with pre-eclampsia if compared with normal matched pregnant women and suggests that the decrease observed is related to placental production.[31] Together, these data suggest that *de novo* synthesized placental melatonin contributes to high maternal blood levels observed during pregnancy, and that this *de novo* synthesized melatonin exerts paracrine, autocrine, and intracrine activities in placental function. Finally, the variation of placental and plasmatic levels of melatonin of preeclamptic patients leaded us to suggest plasmatic melatonin as a biomarker of pre-eclampsia. Yamamoto *et al.* (2013) investigated the effect of four hypertensive diseases during pregnancy on placental melatoninergic system: chronic hypertension, chronic hypertension associated with pre-eclampsia, gestational hypertension and pre-eclampsia.[81] In this study, all four alterations were responsible for the increase of expression of receptor MT1. All three hypertensions increased the expression of tryptophan hydroxylase (TH), but pre-eclamptic placentas demonstrated reduced expression of this enzyme whose role is to initiate the melatonin synthesis pathway by the addition of an OH^- to tryptophan. Despite the analysis of placental MT1 and TH, neither plasmatic nor placental melatonin was analyzed.

Hyperglycemia associated with vascular alterations during pregnancy characterize gestational diabetes mellitus (GDM), and affects between 8% and 17% of pregnancies.[82] This disease stimulates the placental

oxidative stress and affects the feto-placental vascular exchange.[83, 84] Recently, the risk of GDM has been positively associated with polymorphic expressions of the melatonin MT2 receptor. The first study that found this association was performed in a population of Korean ethnic origin. A group of almost one thousand GDM patients without previous diabetes mellitus were selected and two single-nucleotide polymorphisms (SNPs) were identified in MT2 gene and were strongly associated with GDM.[85] Other studies followed this pattern and evaluated the relationship of GDM and SNPs in MT2 gene in different populations. Regardless the population, MT2 gene polymorphisms were strongly associated with GDM.[83] To date, the melatonin effect on GDM is not well described, however the melatonin cytoprotective effect would possibly prevent the alterations caused by GDM.[86]

Appropriate placental development is necessary for the fetal growth and development. Pregnancies with insufficient subsistence usually are related to obstetrics complications, such as fetal growth restriction (FGR). The fetal morbidity associated with this condition is caused by the altered placental development and compromised umbilical blood flow and is extremely high and harmful. The main consequences of FGR are perinatal death, preterm births, low weight at the partum, and retardation in the physical growth and brain development.[87, 88] As the pathophysiology of FGR is related with placental inflammation, hypoxemia and rise of placental oxidative damage, melatonin has been tested in experimental models to alleviate the prenatal effects of FGR. Lemley *et al.*[89] developed an experimental study to evaluate the effects of melatonin on pregnant ewes compromised by FGR. This model was based on the nutriment restriction and demonstrated that oral melatonin was able to increase the uterine blood flow and the fetuses ponderal mass. Based on the placental responsiveness to melatonin treatment, the same group demonstrated that chronic uterine injection of melatonin improves the feto-placental blood flow in pregnant ewes.[90] In accordance with these results, a recent study that mimics FGR demonstrated that injection of melatonin during pregnancy reduces fetal oxidative stress levels, improves neurodevelopment, and enhances cognitive capacity of the lambs.[91]

The disproportion between oxidants and antioxidants in preeclamptic pregnancies leaded to the evaluation of antioxidants as a treatment to this disease. Vitamin C and E were tested as a possible treatment for pre-eclampsia in a vast clinical trial.[92] Despite of theoretical and previous assumptions, the results of the clinical trial demonstrate no beneficial effect of the treatment and even showed decreased birth weight of babies from women treated with vitamin C and E. Based on these results, Milczarek, *et al.* demonstrated that the association of both vitamins C and E with melatonin is extremely effective in the protection of placental mitochondria against the oxidative stress and suggested the combination of this three antioxidants as a possible treatment for pre-eclampsia (Figure 4).[23]

Regardless the concentration and the type of treatment with melatonin, its toxicity is very low. Assays with increased doses of melatonin under chronic and acute treatment have demonstrated that several experimental models are not affected by this indolamine.[93, 94] Moreover, oral treatment with melatonin has generated neither important side effects nor toxic effects.[95]

The toxicity of melatonin was already tested in a variety of experiments that include even experimental models of pregnancy. Oral melatonin was not related to any kind of toxicity during pregnancy of rats and ewes.[94, 96] This fact, in association with the recent demonstrations of its effectiveness against placental abnormalities, resulted in establishing clinical trials that try to correlate the effect of melatonin against alterations as pre-eclampsia and FGR. Hobson, *et al.* initiated a clinical trial (phase I) to evaluate the effect of 30 mg/day of oral melatonin in patients with pre-eclampsia.[97] The length of the treatment begins with the recruitment and is kept until the birth. Several markers of eligible women enrolled in this study are constantly determined, such

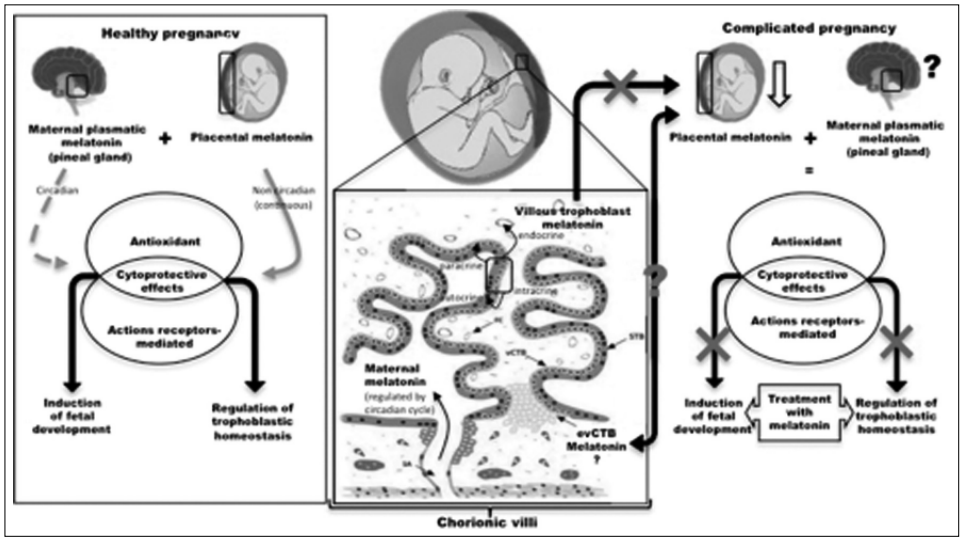

Figure 4.—Potential role of melatonin in healthy and complicated pregnancies. Plasmatic melatonin, released in a circadian manner, reaches the intervillous space and accesses fetal capillaries (FC) through syncytiotrophoblast (STB) and villous cytotrophoblast (vCTB). In parallel, both STB and vCTB produce melatonin locally that can also reach fetal capillaries and maternal circulation. Both plasmatic and placental melatonin are related with maternal and fetal protective effects observed during pregnancy. Although extravillous cytotrophoblast (evCTB) are responsible for the placental implantation in the beginning of pregnancy and may have a relationship with complicated pregnancies, melatonin production in those cells is still unknown. Furthermore, since placental melatonin production during pregnancy abnormalities is compromised, melatonin treatment is suggested to recover the absent protective effects of placental melatonin in those cases.

as plasmatic oxidative stress, markers of pre-eclampsia, markers of maternal morbidity and ultrasound. The same research group that related the treatment of melatonin and the improvement of cognitive capacity of lambs is developing a clinical trial with the aim of determining the effect melatonin treatment on patients with FGR.[98] Similarly to the other clinical trial, this study is a single-arm, open-label study, however the daily treatment in this case is 8 mg/day. Placental, maternal and fetal oxidative stress will be assessed as well as maternal and fetal markers that indicate the possible protection of melatonin against FGR.

Conclusions and future perspectives

Both placental and plasmatic melatonin work synergistically and, despite of the source, this indoleamine has effects that go beyond the endocrine pattern. Maternal melatonin modulates fetal circadian cycle and its development, whereas placental melatonin regulates trophoblast homeostasis and thus placental function and development in a paracrine, autocrine and intracrine manner. Accordingly, despite of its origin, melatonin is a key-factor for successful pregnancy and fetal development.

Research using trophoblast cells is a novel approach to discover the mechanisms by which melatonin plays a pro-survival and cytoprotective role crucial for placental health and, by consequence, for pregnancy well-being and fetal development. Therefore, further researches are needed to better understand the role of the plasmatic and placental melatonin as well as its receptors in normal and abnormal pregnancies. Hence, this will open the way for the development of new preventive and therapeutic approaches in this area.

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ANNEXE 4 – ARTICLE DE REVISION : AN INSIGHT INTO THE ROLE OF CD95 ACROSS THE PREGNANCY.

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Contribution de l'étudiant

Lucas Sagrillo-Fagundes a participé à la validation des sujets de l'article. Il a également rédigé l'article, participé au choix du journal de publication.

AN INSIGHT INTO THE ROLE OF CD95 ACROSS THE PREGNANCY

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Running Title

CD95 across the pregnancy

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Abstract

The fact that CD95 (Fas) is present in female reproductive cells and modulated during the embryogenesis and pregnancy has raised the interest of its role regarding the immune tolerance to the feto-placental unit. CD95 and its ligand, CD95L, have been thoroughly studied due to their role in the immune homeostasis and the elimination of infected and transformed cells. CD95 has been shown as important for the proper embryonic formation and survival. Moreover, altered expression of CD95 or its ligand causes autoimmunity and has also been directly involved in recurrent pregnancy losses and term pregnancy disorders. The objective of this review is to summarize studies that evaluate the mechanisms involved in the activation of CD95 to have an updated global view of its effect on the regulation of the maternal immune system. The immune basis of several pregnancy disorders has an intimate relationship with the modulation of CD95 system components.

Keywords:

CD95, immune tolerance, placenta, embryology.

Introduction

The feto-placental unit is recognized by the maternal immune system as a foreign entity, yet tolerance mechanisms avoid its rejection. Medawar has proposed three hypotheses to explain the tolerance of the pregnancy [1-3]. First, the complete separation between the mother and fetus structures, which was refuted by the comprehension of the hemochorial placentation construction demonstrating the exchange of cellular elements and the direct contact between maternal immune cells and the trophoblasts. The second hypothesis was the lack of immunogenicity of fetal cells, which was ruled out due to the presence of the major histocompatibility complex (MHC) in fetal cells [4]. The third hypothesis stated that the maternal immune system would be tolerant to the fetus per se. The evidence against this theory is that cases of recurrent spontaneous abortions are associated with higher levels of both peripheral NK cells and concentrations of type helper T cell (Th-1) cytokines [5]. Later, the immune privilege of the pregnant uterus was suggested as the main reason for the absence of immune reactions initiation. However, the occurrence of ectopic pregnancies suggests the uterus as a factor, among others, for the maintenance of the pregnancy (Billington 2003). The comprehension of the molecular mechanisms involved in the tolerance of the feto-placental allograft has drawn a comparison to immune-mediated conditions including autoimmunity, transplantation, and cancer. Several modulators of the maternal immune system during the pregnancy have been described, but the precise mechanisms responsible for the immune success of a pregnancy are still vaguely known.

CD95 (also known as Fas) is a membrane receptor that belongs to the tumor necrosis factor (TNF) receptor family. CD95 induces apoptosis [6], inflammation [7], proliferation [8], migration [9], and invasion [10]. With granzyme B/perforin, CD95 is the main system involved in the cell death triggered by $CD4^+$ and $CD8^+$ T cells in allogeneic, transformed, and virus-infected cells [11]. The deregulation of CD95 engenders auto-immunity as observed in patients affected by the lymphoproliferative syndrome type Ia [12, 13]. The actions of the CD95 activation has gathered attention to successful and abnormal pregnancies and will be discussed below.

CD95 functions across the pregnancy

CD95 is mainly expressed in immune cells (including activated B and T lymphocytes, NK cells, monocytes, macrophages) and in the thymus, liver, and placenta [11, 14]. CD95 can be activated by its transmembrane ligand CD95L (FasL/CD178/TNFSF6), soluble CD95L, and agonistic antibodies [15, 16]. Indeed, matrix metalloproteases (MMPs) are able to cleave the extracellular region of membrane-bound CD95L to release a soluble homotrimeric ligand (sCD95L). MMPs are important trophoblastic proteins involved in the invasion into the maternal endometrium during placentation. MMP-7 and MMP-9 for example, have divided their actions into two fronts, they are key factors for the uterine invasion by the trophoblast during the first trimester of pregnancy [17] and are also able to cleave the CD95L [18-20]. sCD95L contributes to the severity of the inflammation and autoimmunity in systemic lupus erythematosus (SLE) patients via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [21] and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathways [22]. However, it is difficult to

predict the biological effect of sCD95L because different cleavage sites exist and for instance, CD95L cleavage by MMP-7 creates an isoform of sCD95L able to induce apoptosis, possibly due to the formation of a multi-aggregated quaternary structure [16, 23]. Indeed, the minimal level of CD95L aggregation required to trigger apoptosis corresponds to a hexamer [24]. Below this aggregation level, sCD95L fails to activate cell death, leading to non-apoptotic signaling activation, responsible for the severity of the disease in autoimmunity and cancer [22].

The apoptotic machinery of CD95 consists of the adaptor protein Fas-associated protein with death domain (FADD), that links CD95 to the pro-caspase-8 upon CD95L stimulation, and initiate the formation of the death-inducing signalling complex (DISC) leading the cascade of caspases activation (Figure 1). In turn, activated caspase-8 cleaves Bid, which moves to the extracellular membrane of mitochondria to initiate the membrane permeabilization and the subsequent release of intermembrane apoptotic factors [25, 26]. The caspase-8 can induce apoptosis with a direct cleavage of caspase-3 in so-called type I cells [27]. On the other hand, type II cells rely on the mitochondria to undergo apoptosis when exposed to CD95L because these cells express higher levels of the X-linked inhibitor of apoptosis protein (XIAP) as compared to type I cells [15]. XIAP binds caspase-3 and prevents its cleavage by caspase 8 [28]. Bid redistribution into the extracellular leaflet of the mitochondria induces the release in the cytoplasm of the second mitochondrial activator of caspases (SMAC), inhibiting the interaction of XIAP with caspase-3 [15, 18] and thereby caspase-3 cleavage. In view of the high levels of XIAP in trophoblasts its CD95-mediated apoptosis might be intimately dependent of the mitochondria [18, 29-32].

CD95-mediated apoptosis has a paramount importance on the apoptosis of autoreactive T cells [33]. Certain organs could possess immune privilege including cornea, brain, and placenta using CD95L expression to repel activated T cells [34]. The serological levels of CD95 and CD95L between 11 and 16 weeks of pregnancy and in the beginning of the third trimester (25 and 30 weeks of pregnancy) are similar to the concentrations of non-pregnant women [35]. CD95L is detected in trophoblasts and Hofbauer cells suggesting a role of CD95 in the placental immune tolerance in pregnancies [36, 37].

Placental trophoblasts are derivatives forms of the blastocyst and differentiate at the very first week of pregnancy. When the adhesion in the uterine decidua is established, trophoblasts differentiate into two pathways: villous and extravillous trophoblasts (vTB and evTB, respectively) [38]. Trophoblasts differentiate into evTB during the first trimester to allow the proper uterine invasion [39-41]. The vTB are composed of the mononuclear villous cytotrophoblasts (vCTB) that differentiate into syncytiotrophoblast (STB), a non-proliferative multinucleated cell that forms the placental layer in contact with the maternal blood [42-45]. evTB cells are classified in two morphological phenotypes: proliferative, located on the base of the anchoring villi comprised in several layers of cells; and invasive, with cells able to invade the endometrium (Figure 2) [46-48]. Cells showing the invasive phenotype express high levels of CD95L in both first and third trimesters [49, 50]. Cells with the proliferative phenotype from term placentas also present CD95L at the, but lower compared to the invasive evTB [50].

During the first trimester of pregnancy, the invasive evTB invade the uterine vessels and replace the endothelial maternal cells to accommodate the increase in blood flow that is needed to support the dramatic fetal growth across the pregnancy [41, 47, 51, 52]. The replacement of endothelial cells by invasive evTB is mandatory to increase the oxygenation in the placental villi and avoid dysfunctions related to intermittent oxygen levels during the pregnancy, such as preeclampsia, intrauterine growth restriction and preterm birth [53-55]. Several details about the replacement of maternal endothelial cells by evTB are still unknown, however, *in vitro* and *ex vivo* studies suggest that the apoptotic signal necessary to trigger the replacement delivered by the invasive evTB [56-59]. The uterine endothelial and the smooth muscle cells express CD95, which participates in the remodeling of the uterine vessels. Using an *in vitro and a* spiral artery explant model*,* primary human trophoblasts pre-treated with a neutralizing anti-CD95L antibody fail to induce apoptosis in endothelial and smooth muscle cells. Similarly, depletion of CD95L from primary trophoblast-conditioned medium also abrogated the activation of apoptosis in smooth muscle cells [51, 60].

Hofbauer cells are macrophages located in the placental stroma, likely derivatives from fetal hematopoietic cells [61]. These cells present high cell count during the first trimester, with a role on the tissue remodeling, immunosuppression, and promotion of Th-2 response [61, 62]. The interplay between trophoblasts and Hofbauer cells favors the phagocytic capacity and shifts profile of cytokines of the latter to Th-2 with increased interleukin (IL)-10. The transforming growth factor beta (TGF-β) signaling pathway is a putative responsible for the trophoblastic modulations on macrophages. The inhibition of TGF-β in trophoblasts leads to the downregulation of the Th-2 response but doesn't affect the phagocytic capacity [63-65]. The increased expression level of CD95L in Hofbauer cells has been thought to be associated with the skewing of the cellular profile toward pro-inflammatory responses and poor pregnancy outcomes, such as preeclampsia [63, 66, 67].

A series of in vitro and ex vivo studies have suggested the lack of CD95L on the apical and basal membranes of the syncytiotrophoblast. Instead, CD95L is rather accumulated within cells and then released in exosomes [68-70]. The exact exosomal location of CD95L (lumen or surface) is still controversial, and could be related to variations of regarding the adopted techniques (i.e. villous explants *vs.* primary trophoblast cells). Regardless of its exact location, syncytial exosomes encompass the full-length form of CD95L, which can induce apoptosis in T-lymphocytes cells. Moreover, supernatants without exosomes do not induce apoptosis, confirming that the exosomal CD95L and not its metalloprotease-cleaved counterpart is responsible for the death of activated lymphocytes from healthy donors (Table 1) [68, 70-72].

Cancer cells resist to CD95-mediated apoptosis through a variety of strategies including over-expression of the short isoform of cellular FLICE Inhibitory Protein ($cFLIP_S$) [73], an inhibitor of the interactions of caspase-8 with the DISC, or reduced expression of FADD [74] or caspase-8 [75, 76]. The possibility of down-regulation of CD95 as a mechanism to bypass the apoptotic pathway has been described in the placental choriocarcinoma cell lines BeWo and NJG. In fact, choriocarcinoma cells express CD95L, a mechanism that could enhance their survival capacity via induction of CD95-mediated

apoptosis of immune cells, thus mediate their metastatic capacity towards highly vascularized organs [77-79]. The CD95 system is differently modulated in cancer and normal human trophoblast cells [80-82]. p53, which induces the translocation of CD95 from the Golgi complex to the cell membrane, is inhibited by the transcription factor called Factor that Binds to the Inducer of short transcripts of HIV-1 (FBI-1) [83]. Placental choriocarcinoma cells, among other gynecological malignancies, express high levels of FBI-1 [84-86]. Compared to normal trophoblasts, the FBI-1 over-expression presents two distinct actions that contribute to the uncontrolled tumoral growth: (1) in trophoblastic malignancies, FBI-1 enhances the activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway via phosphorylation of the serine 473 , leading to cell migration and invasiveness; and (2) FBI-1 is able to inhibit the mRNA expression of apoptotic inducers including CD95 and caspases-8, -9 and -10 via repression of the p53 pathway [81-83, 85, 87].

First, second, and third-trimester villous trophoblasts express CD95 and CD95L, indicating their resistance to CD95-mediated apoptosis [35, 36]. It has been shown, for example, that TNF, but not CD95L induces apoptosis in term or first-trimester villous cytotrophoblasts or in term syncytiotrophoblast [14]. A possible protection against the CD95-mediated apoptosis would be mediated by the basal trophoblastic nitric oxide (NO) production. Indeed, the treatment with growth factors that stimulate endogenous NO was found to enhance the trophoblastic survival [14, 88]. Likewise, the NO is responsible for the absence of response to CD95L in a human extravillous trophoblast-derived cell line (SGHPL-4). While the levels of NO are stable, there is a lack of CD95L effects on the cell survival, but, in absence of NO, the levels of cell death increase by more than 50% [89]. The inhibition of NO production leads to rapid formation of CD95 clustering, a crucial and initial step in the activation of the apoptotic signaling pathway [88, 90]. The molecular involvement of the NO inhibition on the CD95-mediated apoptosis is apparently indirect [91], via the inhibition of the protein kinase C ε (PKCε) activity; leading to the recruitment of c-FLIP to DISC, to finally block the initial steps of the apoptotic signal [88, 89]. Aschkenazi and colleagues investigated the influence of the balance between the Th-1 pro-inflammatory cytokines and the Th-2 anti-inflammatory cytokines on the destiny of trophoblasts. While TNF and interferon-gamma (Th-1 cytokines) induce the expression of CD95 and promote the CD95-mediated apoptotic signaling pathway IL-10, leads to the increase in FLIP expression, which in turn blocks the CD95-mediated apoptotic cue in A3 and HTR/8 trophoblastic cells [92].

The decoy receptor 3 (DcR3) is a member of the TNF-R superfamily devoid of a transmembrane domain. It shares sequence homology with CD95 (17%) [93]. This receptor was first identified in human cancer cells but has also been detected in monocytes and dendritic cells of silicosis, bacterial infection and systemic lupus erythematosus patients [94-96]. DcR3 is able to bind and neutralize CD95L and two other members of the tumor necrosis factor superfamily ligands, LIGHT (CD258/TNFSF14) and TNF-like molecule 1A (TL1A/VEGI/TNFSF15) [97, 98]. DcR3 seems to interfere with the CD95 / CD95L interaction and, thus inhibit the CD95-mediated signals. Therefore, its overexpression might exert a mechanism of protection against the cytotoxic effects of CD95L [93, 99, 100]. Regarding the feto-placental environment, DcR3 transcript was detected in the choriocarcinoma cell line JEG-3, in human term Hofbauer cells and in

the human primary trophoblasts from the first and third trimester. Quantification of the western blot data showed a higher level of DcR3 in the uterine decidua of normal pregnancies compared to those of anembryonic pregnancies [101-103]. In sum, the concept of immune privilege in successful pregnancies might be associated with the different mechanisms involved in the modulation of the CD95-system activation.

CD95 during the embryonic development

Makrigiannakis, *et al.* (2001) suggested the CD95L immunomodulatory effects could participate in the embryo implantation via the corticotropin-releasing hormone (CRH) [104]. This hormone exerts a control in the hypothalamic-pituitary-adrenal axis and in the female reproductive system. CRH stimulates inflammation, which is necessary in the uterine embryo implantation [105]. To avoid the immune rejection following the implantation, the trophoblasts produce CRH, which, via autocrine effect, up-regulates the expression of CD95L, leading to the apoptosis of the maternal T cells [106, 107]. The receptor CRH-1, which triggers the release of the corticotropin in stressful situations, is expressed in both maternal decidua and evTB with invasive phenotype [108]. Female rats treated with the antagonist of CRH-1, antalarmin, had lower ratios of successful implantation, live embryos and lower levels of CD95L in the innermost region close to the embryo. The cytotoxic effect of CD95L on T cells was evident due to the fact that the fertility of immunodepressed animals lacking T cells was not affected by antalarmin treatment [104, 109]. In association with the evTB, the uterine epithelial cells and differentiated endometrial stromal cells also produce CRH that leads to CD95L expression and the control of the maternal immunity during the pregnancy [110]. The CRH-driven CD95L expression has also been described in cancer cells. Indeed, the up-regulation of CRH and CD95L is found in higher tumor stages of ovarian cancer, emphasizing the immune privilege generated by CD95L [111, 112].

Considering the complexity of the immune tolerance necessary for a successful pregnancy, several pathways act synergistically. The disrupted CD95/CD95L interaction in mice has been linked to poor pregnancy and the fetal development outcomes [113]. The mouse strain *gld (*generalized lymphoproliferative disease) presents a point mutation (T to C) near C-terminus of CD95L, which dramatically reduces its affinity to CD95, present in the autoimmune pathology autoimmune lymphoproliferative syndrome (ALPS) type Ib [114, 115]. Homozygous mating *gld* mice developed necrosis and extensive leukocyte infiltration at the decidual-placental confluence at mid-gestation. Viable and fertile offspring were delivered, although less numerous, due to the extensive intrauterine death likely caused by the absence of placental immune privilege, granted by the active CD95L. [113, 116] The intrauterine death of *gld* mice corroborates the data of massive death of mice homozygous for caspase-8 deletion at mid-gestation. It has been shown that the formation of the heterodimer caspase-8 – FLIP supresses the activation of the necroptic signal following TNF ligation and also could be involved in non-apoptotic roles, such as cell migration and activation of NFκB [117].

Mating between CD95L homozygous knockout mice produced a normal number of offspring and sex ratio, indicating that the null allele of *CD95L* did not affect fecundity. This strain demonstrates however early death (< 4 months of age) of 50% of the homozygous mice due to massive splenomegaly, severe

glomerulonephritis, and lymphadenopathy [118]. Mice bearing mutated or abrogated versions of the *CD95L* gene have their reproductive status negatively affected; yet the reason for the variation of the onset of the clinical evidences is still unknown (Table 2).

In endometriosis and unexplained infertile patients, increased levels of soluble CD95L are detectable in the maternal serum and also in the follicular fluid, the microenvironment necessary for the oocyte development. Correspondingly, the activity of antioxidant enzymes is decreased in both serum and follicular fluid of IVF patients compared to the control group [119]. A systematic review suggests that metformin, majorly used as a treatment for type-2 diabetes [120, 121], may enhance the IVF success due to the regulation of ovulation, reduction of the CD95L levels in the follicular fluid and the DNA fragmentation of oocytes [119, 122-124]. During the last couple of decades, Gonadotropin-releasing hormone (GnRH) antagonists have been added to IVF protocols due to reduce the incidence of severe ovarian hyperstimulation syndrome [125]. However, it has been also associated with less successful embryo implantation and live birth rates, mainly caused by lower endometrial receptivity [126]. A possible cause for this failure could be the fact that CD95L protein expression is increased in the endometrium of patients treated with GnRH antagonists, concomitant with higher levels of 17β-Estradiol, responsible for up-regulation of *CD95L* mRNA and cytotoxicity in NK cells [127, 128]. Melatonin has also been used to improve the ratio of successful implantations in IVF procedures. This molecule is largely known for the regulation of the sleep-awake cycle via pineal production. It is also synthesised by other organs, including placenta, which metabolises melatonin across the whole pregnancy with higher levels at the delivery moment. [124, 129-133]. The deeper investigation of the effect of melatonin on CD95 in trophoblasts, macrophages and T cells could lead to a better comprehension of the mechanisms activated for the cell survival/apoptosis during the pregnancy [132, 134]. Melatonin is known to present distinct sets of modulations, with a protective profile for normal cells and a toxic profile for cancer cells. The differential activation of downstream pathways of the melatoninergic specific membrane receptors could be directly involved in its dual effects [135]. As recently reviewed, melatonin can modulate the axis of action of T cells from SLE patients to shift the cell fate to the Th-2 profile [132, 136].

CD95: Pregnancy and autoimmunity

The possibility of becoming pregnant had been a critical situation for autoimmune disease patients, due to the possible disease worsening and due to poor gestational outcomes. This context has been changing with maternal counseling, precise detection of the type of autoimmune disease, and with the replacement of teratogenic for safer medication that avoids the flare of the symptoms during the pregnancy [137-141]. Autoimmune diseases are negatively correlated with fecundity success; indeed, pre-clinical stage autoimmune patients (asymptomatic and even unaware of their disease) also have decreased fecundity success. With the implantation established, lower tolerance towards the embryo leads to more frequent early spontaneous miscarriage. Finally, term pregnancy disorders are also intimately linked with excessive immune response [142, 143]. This is in line with the immunity activation in cases of infections, which lead to excessive immunologic reactions, directly related to preterm birth and

shallow placental invasion [144]. Because deregulation of CD95/CD95L leads to autoimmunity, the modulation of the CD95 system in pregnant women bearing an autoimmune disease has been analyzed in in the multiple sclerosis. Despite the variations in the immune counts, CD95L and soluble CD95 do not differ between pregnant women with and without multiple sclerosis before, during, and after the pregnancy [145-148]. The real impact of the autoimmunity on perinatal disorders remains controversial and the deeper comprehension of the role of CD95 and other protagonists on the autoimmunity during the pregnancy could propose new molecular targets to tackle idiopathic infertility and recurrent pregnancy loss. The insights related to poor pregnancy outcomes and effects on the offspring health in mice models that mimic CD95 health conditions are possible indications to discover the underlying role of CD95 on the female reproductive system [149-151].

Role of CD95 system on early pregnancy losses

Three or more pregnancy losses in a row characterize the recurrent pregnancy loss (RPL). Intriguingly, immunohistochemistry of the uterus of RPL patients, in comparison to control group, demonstrated no variation of CD95L, but lower levels of CD95 expression and activation of caspase-3 [152, 153]. The lower presence of CD95 in the uterus of RPL patients is associated with decreased apoptotic signaling in the uterine decidua during the implantation phase of the pregnancy, rendering the uterus less responsive to the establishment of the placental invasion [154]. On the other hand, the placental tissue of RPL patients shows increased expression of CD95 CD95L and apoptosis ratio 3-times higher, in comparison to the first trimester placentas of voluntary abortion cases. PKC, which is an inhibitor of the CD95-mediated apoptosis, presents lower expression in RPL placentas in comparison to the control [155]. Moreover, co-culture of decidual lymphocytes from RPL patients with EVT-choriocarcinoma hybrid cells (clone AC1M88) induces the apoptosis of the latter, whereas the presence of a neutralizing anti-CD95 antibody abolishes this process [155-157]. Guenther, et al. have described an increased population of maternal macrophages in association with increased trophoblastic apoptosis in placentas of spontaneous miscarriages, likely due to the increased expression of CD95L [158]. Even if some protagonists have been identified, the mechanisms involved in the deregulation of the CD95 system remain to be elucidated and coincide with the fact that the real scenery behind early pregnancy losses is still vague. [63].

Role of CD95 system on term abnormal pregnancies

Preeclampsia is the most common cause of fetal mortality; 500,000 infants die every year due to this condition [159-161]. The pathogenesis of preeclampsia is still a matter of debates. Redman and Sargent have divided the disease into two sections: the placental and the maternal activation. The former consists in the shallow trophoblastic invasion and improper remodeling of the spiral arteries of the uterine decidua, leading to inadequate blood flow into the intervillous space and onset of erratic hypoxic conditions. The latter is the maternal positive feedback response to the local placental disorder, which leads to a systemic inflammatory overreaction related to renal and cardiac overcharge, maternal seizure, preterm birth, and intrauterine growth restriction [162-164]. Preeclampsia shares many actors involved in the early pregnancy

losses, including the CD95 system [162, 165-167]. The H8 trophoblast cell line has its viability reduced when exposed to 10% of serum from preeclamptic patients and addition or a neutralizing anti-CD95L restores its cell viability [168, 169].

The expression of the receptor CD95 in cord blood samples and in T-cells is not modulated by preeclampsia [170-173]. Preeclampsia is caused by the shallow evTB invasion, possibly due to the loss of placental protection against the maternal immunity. Unfortunately, preeclampsia diagnosis is only possible from the 20th week of pregnancy, via the detection of maternal symptoms. At this stage of the pregnancy, the placental invasion should already be established, but the preeclampsia effects irretrievably impair it [40, 165]. The membrane-bound CD95L is reduced in placentas of preeclamptic women, whereas its soluble counterpart is elevated in these patients, if compared to healthy subjects [171, 173-175]. The accumulative data with divergent conclusions about the modulation of the CD95 system in preeclampsia is likely due to small groups, diagnostic criteria, tissue investigated and genetic distinctions of population investigated. Regardless, the improper placental invasion, described in *gld* mice leads to necrosis in the uterine decidua and poor pregnancy outcomes, with strong similarities with preeclampsia [113, 176-178].

HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome, a variant of severe preeclampsia, is responsible for high maternal and perinatal mortality [179, 180]. HELLP patients demonstrate increased levels of soluble and membrane-bound CD95L in sera and in villous cytotrophoblasts, respectively compared to patients with milder preeclampsia and controls [174, 181]. HELLP patients possess growing levels of a multimeric form of sCD95L, with higher molecular weight (90–130 kD), released in microvesicles and able to induce CD95-dependent trophoblastic apoptosis [181-183]. CD95 and sCD95L have also been measured in the amniotic fluid of pregnancies with small, appropriate and also large fetuses for their gestational age. CD95 was not modulated according to the fetus size. The sCD95L levels, though, were increased in severe small gestational age fetuses (≤5th centile), condition intimately related with poor pregnancy outcomes and long-term deleterious consequences [184, 185].

To mimic in a rat model the intrauterine growth restriction, Belkacemi et al. have restricted in 50% the food income of dams during the second half of the pregnancy. As expected, maternal, placental and fetal weights were significantly reduced. In concomitance, the rat placental zones basal and labyrinth, respectively responsible for the hormone production and nutrient transfer, demonstrated increased expression of the whole machinery involved in the CD95-mediated apoptosis. [186]. A recent study demonstrated as steady the expression of CD95 in the placenta of intrauterine growth restriction patients. The expression of CD95L, though, is significantly increased in comparison with the control group and with a group of patients of intrauterine growth restriction caused by preeclampsia [173]. Finally, gestational diabetes mellitus leads to large for gestational age fetuses and larger placenta, due to lower apoptosis activation in trophoblasts, possibly due to down regulation of the placental CD95 and CD95L protein expression, associated with increased amounts of anti-apoptotic factors (Table 3) [186, 187].

Perspectives: New insights about the role of CD95

Placental and tumor-related tissues have a common asset - CD95 modulation - in parallel with several other immune factors also involved directly or indirectly [139, 188]. A recent study enrolled a pregnant subject bearing breast cancer to investigate the expression of overlapped genes in the uterus, breast and lymph node, considered self tissues, compared to placenta, breast cancer tissue, and tumor bearing lymph node, considered non-self tissues [189]. Indoleamine 2,3-dioxygenase (IDO) is an enzyme responsible for catabolizing tryptophan into its metabolite kynurenine usually upregulated in tumor microenvironments. IDO expression was increased in all non-self tissues, compared to their analogous. The lack of tryptophan is responsible for reducing the proliferation and activity of T cells, additionally, overexpression of IDO triggers T cells apoptosis via CD95-mediated apoptosis [190, 191]. Regarding the importance of IDO during the pregnancy, its lower expression in the placental tissue is correlated with both preeclampsia and recurrent pregnancy losses [192, 193]. Other similarity between the non-self tissues is the upregulation of the programmed cell death protein ligand-1 (PD-L1) in placental and breast cancer tissues, which is able to extinguish the immune response [194, 195]. This case report presents the “placental immune-editing switch”, a theory that tries to establish similarities between the abilities of placental and cancer cells to develop mechanisms to avoid the immune rejection and promote immune tolerance based on similar patterns of genetic and immune modulation [189, 196, 197]. The investigation of similar cases is necessary to improve the strength of these observations and to highlight additional genes or pathways involved in cancer and pregnancy immune privilege.

The immune privilege observed in successful pregnancies is not only related to placental cells; it is intimately based on the plasticity of the maternal T cells according to the function and mediators released. As previously reviewed [198, 199], the fine balance of the Th-1/Th-2 immunity with a dominance of the Th-2-type immunity is required for successful pregnancies. The lineage of T cells Th-17 (which selectively produces IL-17) has been related with pathogenesis of autoimmune diseases and rejection [199, 200]. Different studies have found conflicting data regarding the Th-17 levels in the human uterine decidua in normal pregnancies [198, 201, 202]. In both serum and uterine decidua of RPL patients, though, Th-17 and Il-17, which mediates tissue infiltration and the expression of pro-inflammatory cytokines, is importantly increased compared to voluntary abortion cases [203]. The regulatory T (Treg) cells are also active players of the fine control of immune tolerance of the pregnancy. This subset of T cells is the responsible for controlling the excessive effector immune response via expression of anti-inflammatory cytokines such as IL-10 and TGF-β [204, 205]. It is known that, in successful pregnancies, similar to the Th-1/Th-2 ratio, Th-17/Treg shifts in favour of the Treg [199]. In contrary, in cases of RPL and preeclampsia, Th-17/Treg is destabilized with increase in the uterine decidua of Th-17 and Il-17 and rather similar levels of Treg cells [203, 206]. cl-CD95L is able specifically to induce the trafficking of Th-17 but not Treg cells towards inflamed organs and induce the activation of pro-inflammatory responses in the heterozygous MRL.*FAS*$^{lpr/+}$ mouse model that expresses lower levels of CD95. This animal model presents SLE-like symptoms but lack of sensitivity to CD95-mediated apoptosis [7, 207]. Very interestingly, the levels of cl-CD95L are increased in

RPL patients, which could imply that its non-apoptotic pathways could also be one of the factors involved in the increase in the trafficking of Th-17 T cells to the uterine decidua and tissue damage [22, 207].

Conclusion

Clinical trials have been conducted with possible treatments to improve the rate of successful pregnancies. The complexity involved at the modifications of the maternal anatomy, physiology, epigenetics, and immunology during the pregnancy demonstrates the huge challenge involved in the research of reliable treatments for poor outcome pregnancies. Indeed, researches involving subsets of populations have worked, the extrapolation, however, has generated less promising results. A molecular comprehension of the mechanisms like CD95, involved in the pregnancy-field progression, is useful not only for the obstetric field; it can be useful to other domains also modulated by the CD95-system, such as oncology and immunology.

Author contributions

All co-authors contributed to the decision of each section of the manuscript LSF, was actively involved in the writing of the manuscript. JBP contributed to the writing and created both figures of the manuscript. CV and PL assisted in drafting the manuscript. Finally, all co-authors provided critical revisions and provided their final approval for the manuscript's publication in Human reproductive update. This manuscript is not under review elsewhere and all authors are in agreement with the contents of this manuscript.

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Tables

Table 1. The presence and actions of the CD95-system in cells involved in the pregnancy

Cell type	Genotype	CD95	CD95L	Cell actions during the pregnancy	CD95-system involvement	Reference
T-lymphocytes	Maternal	+	+	Rise of the immune response to fetal antigens	Induction of cell death of cells bearing alloantigens	Stenqvist et al. 2013
Uterine endothelial cells	Maternal	+	-	Materno-placental blood exchange.	CD95 mediated apoptosis. Replaced by invasive evTB	Ashton et al. 2005
Smooth muscle cell	Maternal	+	-	Materno-placental blood exchange.	CD95 mediated apoptosis. Replaced by invasive evTB	Harris et al. 2006
Hofbauer cells	Fetal	+	+	Involved in the success of the placental vasculogenesis	Control the uterine invasion	Zorzi et al. 1998
Proliferative evTB	Fetal	+	+	Proliferative cells which differentiate into invasive evTB	Avoids the cell death mediated by maternal T-lymphocytes	Murakoshi et al. 2003
Invasive evTB	Fetal	+	+	Placental invasion and replacement of uterine endothelial cells	Avoids the cell death mediated by maternal T-lymphocytes	Murakoshi et al. 2003
vCTB	Fetal	+	+	Differentiation and renewal of the syncytiotrophoblast.	Avoids the cell death mediated by maternal T-lymphocytes	Zorzi et al. 1998
Syncytiotro-phoblast	Fetal	+	+	Outer layer of the placental barrier; direct contact with the maternal serum.	Avoids the cell death mediated by maternal T-lymphocytes	Zorzi et al. 1998

evTB: Extravillous trophoblasts vCTB: villous cytotrophoblast

+ : structure present in the cell

- : structure absent in the cell

Table 2. Description of the fertility status of mouse strains with altered *CD95L expression*

Mouse strain	Suitable model	Fertility outcome and effects	Reference
Gld* homozygous (SNP of *CD95L)	Systemic Lupus Erithematous-like phenotype	- Less numerous offspring - Necrosis and extensive leukocyte infiltration of the placenta	Hunt et al. 1997
***CD95L* KO**	Autoimmune lymphoproliferative syndrome-like phenotype	- Normal number of offspring and sex ratio - 50% of Early death due to splenomegaly, glomerulonephritis, and lymphadenopathy	Karray et al. 2004

Table 3. Modulation and effects of the CD95 on pregnancy-related abnormalities

Pregnancy-related abnormality	CD95	CD95L	Effects and Outcomes			Reference
			Maternal	Fetal	Placental	
Infertility	↑ T cells	↑ serum	↑ Cell free DNA	Poor implantation	N.A	Guan et al. 2016
Recurrent pregnancy loss	↓ uterus	↑ T cells and macrophages	Shift for Th-1 cytokine profile	1st trimester miscarriage	↑ CD95-mediated apoptosis	Brown et al. 2014
Preeclampsia	↔ T cells	↑ serum ↓ placenta	Inflammatory overreaction	Poor pregnancy outcomes	↑ CD95-mediated apoptosis	Resic Karara et al. 2016
HELLP	↔ T cells	↑ serum ↑ placenta	Hepatic failure caused by CD95L	High mortality	↑ CD95-mediated apoptosis	Prusac, Zekic Tomas, and Roje 2011
IUGR	↔ amniotic fluid	↑ amniotic fluid	Inflammatory overreaction	Severely small fetus	↑ CD95-mediated apoptosis	Vrachnis et al. 2013
Gestational Diabetes	↓ placenta	↓ placenta	↑ body weight	Macrosomia	↓ CD95-mediated apoptosis	Magee et al. 2014

↑ increase in the cell or tissue

↓ decrease in the cell or tissue

Figures

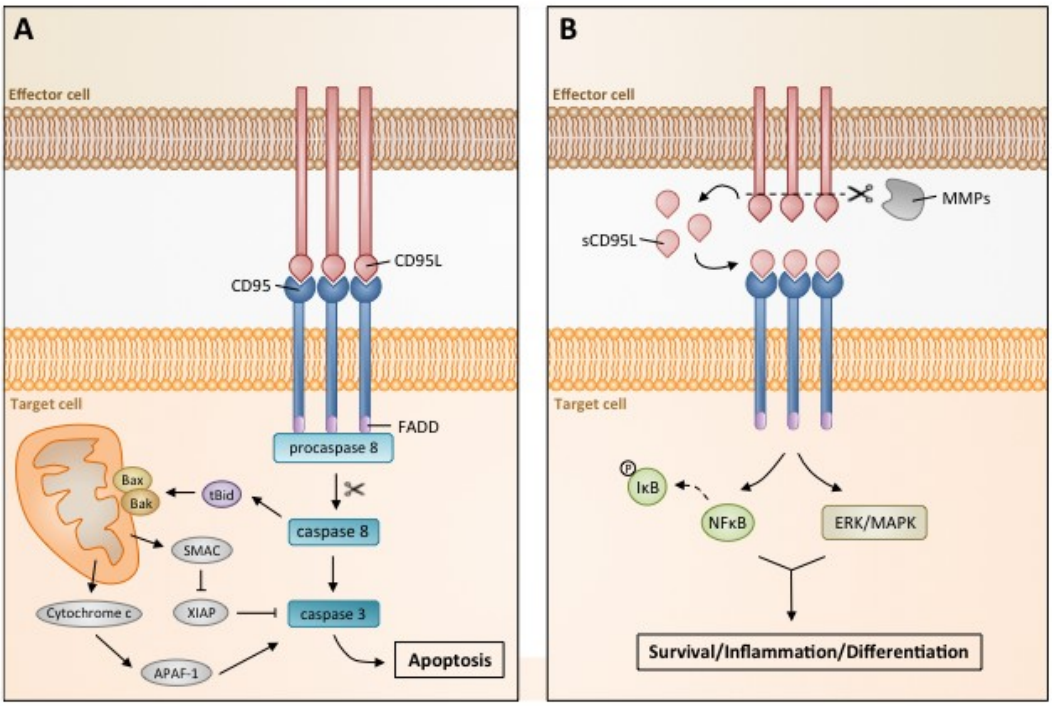

Figure 1. Signaling pathway of CD95 and CD95L. (A) Membrane-bound CD95L activates the CD95, which recruits the Fas-associated protein with death domain (FADD), to induce the procaspase 8 cleavage into active caspase 8. In turn, caspase 8 allows the activation of apoptosis directly via cleavage of downstream caspases, such as caspase 3 or via the disruption of the mitochondrial permeability, mainly described in type I cells. In type II cells, the cleaved-caspase 8 triggers the cleavage of Bid into truncated (tBid), which triggers the permeabilization of the mitochondrial outer membrane. Due to this permeabilization, both cytochrome C and second mitochondrial activator of caspases (SMAC) are released from the mitochondria. Cytochrome c release activates the apoptotic peptidase activating factor 1 (APAF-1), which cleaves the caspase 3 to induce the CD95-mediated apoptosis. On the other hand, SMAC inhibits the binding of the X-linked inhibitor of apoptosis protein (XIAP) to caspase 3; consequently, the inhibition of the caspase 3 by XIAP is prevented. (B) The cleavage of the extracellular region of CD95L (sCD95L) by metalloproteinases (MMP's), such as MMP3, MMP7, and MMP9, binds the CD95, which, in this case, induces the activation of downstream pathways responsible for the modulation of the transcription factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinase (MAPK) pathways.

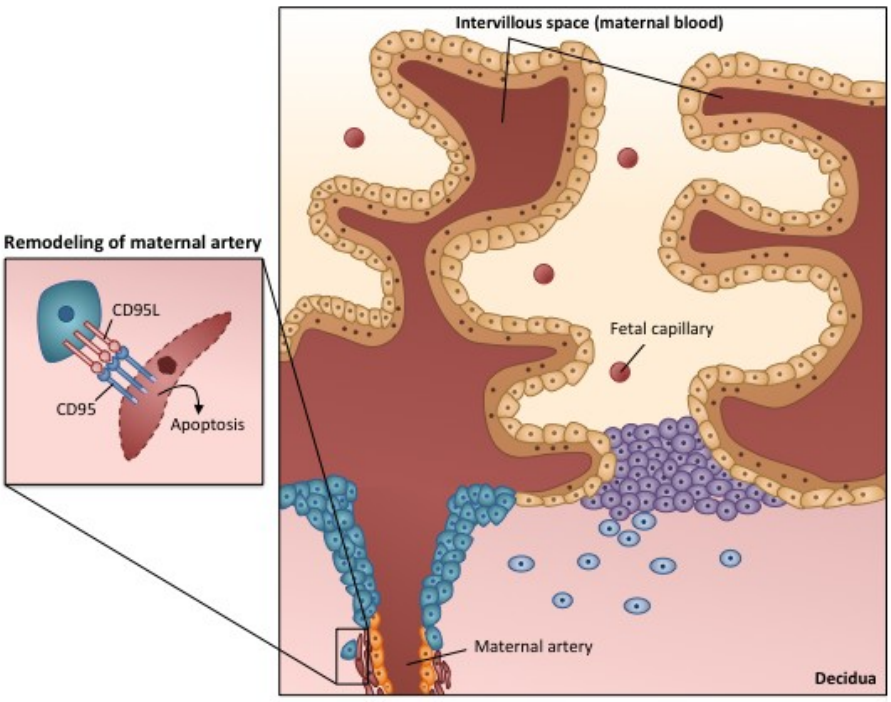

Figure 2. Functions of CD95 and CD95L during human placentation. The presence of CD95L in the proliferative (purple) and invasive (blue) extravillous trophoblasts allows the apoptosis of both smooth muscle (red) and uterine endothelial cells (orange), which bear the CD95 receptor. The apoptosis of smooth muscle and endothelial cells results in the remodeling of the maternal artery, important for the placentation.