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EFFET PROTECTEUR DE LA MÉLATONINE SUR L'HOMÉOSTASIE DU TROPHOBLASTE VILLEUX : IMPLICATIONS DANS LA GROSSESSE NORMALE ET COMPLIQUÉE PAR LA PRÉÉCLAMPSIE

Caractérisation de la mélatonine dans le placenta humain

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EFFET PROTECTEUR DE LA MÉLATONINE SUR L'HOMÉOSTASIE DU TROPHOBLASTE VILLEUX : IMPLICATIONS DANS LA GROSSESSE NORMALE ET COMPLIQUÉE PAR LA PRÉÉCLAMPSIE

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RÉSUMÉ

La mélatonine possède une importante action cytoprotectrice dans plusieurs tissus de l'organisme. En outre, des études suggèrent qu'elle jouerait un rôle protecteur au niveau du placenta. Mes travaux de maîtrise ont démontré que les récepteurs MT1, MT2 et RORα de la mélatonine sont exprimés dans le tissu placentaire humain normal à terme, sans toutefois préciser le type de cellules les exprimant. Les taux sanguins maternels de mélatonine augmentent significativement tout au long de la grossesse, la source de cette augmentation est inconnue, et diminuent dans les cas de prééclampsie (hypertension de grossesse associée à une protéinurie). Cette complication obstétricale affecte 3 à 15 % des grossesses et représente une importante cause de mortalité maternelle et périnatale. Un état d'hypoxie/réoxygénation (H/R) placentaire, induisant une altération de l'équilibre pro-oxydant-antioxydant, est à la source de l'augmentation de l'apoptose mitochondriale du syncytiotrophoblaste caractérisant la pathogénèse de la prééclampsie.

Notre **hypothèse de recherche** est que la mélatonine joue un rôle protecteur dans le maintien de la survie du trophoblaste villeux, essentiel au bon déroulement de la grossesse, et qu'une altération de la signalisation ou de la production placentaire de mélatonine est présente chez la grossesse compliquée par la prééclampsie et peut ainsi participer à sa pathogénèse. Pour vérifier cette hypothèse, nous avons répondu aux trois **buts** suivants : **(1)** Est-ce que le placenta humain produit, *de novo*, de la mélatonine et est-ce que les différents types de cellules trophoblastiques expriment ses récepteurs? **(2)** Est-ce que la mélatonine peut prévenir l'apoptose mitochondriale du syncytiotrophoblaste induite par de l'H/R *in vitro*? et **(3)** Est-ce que l'expression et/ou l'activité des enzymes responsables de la synthèse placentaire de la mélatonine ainsi que l'expression de ses récepteurs sont altérées dans les placentas de grossesses compliquées par la prééclampsie par rapport à la grossesse normotensive?

Les résultats de cette étude ont démontré que dans le placenta humain à terme, le trophoblaste villeux produit de grandes quantités de mélatonine et exprime ses récepteurs. Ils montrent également que par une action autocrine/paracrine, la mélatonine prévient l'augmentation de l'apoptose mitochondriale et maintient l'équilibre pro-oxydant-antioxydant chez le syncytiotrophoblaste soumis à une H/R. Enfin, ces travaux démontrent une diminution significative de la production de mélatonine et de l'expression de ses récepteurs dans les placentas de grossesses compliquées par la prééclampsie en comparaison à la grossesse normotensive. La mélatonine possède donc une action bénéfique pour promouvoir la survie du syncytiotrophoblaste, qui est diminuée dans la prééclampsie.

En conclusion, cette étude montre un premier rôle autocrine/paracrine de la mélatonine dans le maintien de l'homéostasie du syncytiotrophoblaste dans la grossesse normale et propose que la mélatonine puisse prévenir l'augmentation de l'apoptose du syncytiotrophoblaste impliqué dans la pathogénèse de la prééclampsie. Dans l'ensemble, ces travaux ont permis de mieux cerner le rôle de la mélatonine dans le placenta humain en situation normale et dans la grossesse compliquée par la prééclampsie, et d'ouvrir la voie à un nouveau biomarqueur potentiel de la prééclampsie et d'établir la mélatonine comme une nouvelle piste pour prévenir ou traiter la prééclampsie.

Mots clés : mélatonine / récepteur de la mélatonine / antioxydant / placenta / trophoblaste villeux / syncytiotrophoblaste / hypoxie/réoxygénation / apoptose mitochondriale / stress oxydatif / prééclampsie

À mon grand-père qui n'a jamais abandonné et dont le courage a été exemplaire jusqu'à la fin. Tu m'as appris deux grandes choses, ne jamais baisser les bras et pêcher.

Roch Lemire 1929-2011

On fait la science avec des faits, comme on fait une maison avec des pierres, mais une accumulation de faits n'est pas plus une science qu'un tas de pierres est une maison.

Henri Pointcaré

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AANAT	Arylalkylamine N-acétyltransférase
ABTS	2,2'-azino-bis (3-éthylbenzthiazoline-6- acide sulfonique)
ADN	Acide désoxyribonucléique
AFMK	N^1-acétyl-N^2-formyl-5-méthoxykynuramine
AIF	Facteur induisant l'apoptose
Akt	Tyrosine kinase A
AMK	N^1-acétyl -5-méthoxykynuramine
AMPc	Adénosine monophosphate cyclique
Apaf-1	*Apoptosis protease activating factor 1*
ARN	Acide ribonucléique
Bad	Protéine B associée à Bcl-2
Bax	Protéine X associée à Bcl-2
Bcl-2	Lymphome à cellules B-2
CAT	Catalase
CRH	Corticolibérine
Cu	Cuivre
Cyto c	Cytochrome c
EC	Cellule endothéliale
EGF	Facteur de croissance épidermique
ELISA	Dosage enzymatique par immunoadsorption (*Enzyme-linked immunoserbent assay*)
EndoG	Endonucléase G

evCTB	Cytotrophoblaste extravilleux
FC	Capillaire fœtal
FF	Fibroblaste fœtal
FSH	Hormone folliculo-stimulante
LPTC	Grande cellule polygonale trophoblastique
GC	Cellule géante trophoblastique
GM-CSF	Facteur stimulant les colonies de granulocytes et de macrophages
GnRH	Gonadolibérine
GPx	Glutathion peroxydase
hCG	Hormone gonadotrophine chorionique humaine
HERV-W	Rétrovirus endogènes humains W
HERV-FRD	Rétrovirus endogènes humains FRD
HIF	Facteur induit par l'hypoxie
HIOMT	Hydroxyindole O-méthyltransférase
H_2O_2	Peroxyde d'hydrogène
hPL	Hormone lactogène placentaire
H/R	Hypoxie/réoxygénation
IAP	Protéine inhibitrice de l'apoptose
i-evCTB	Cytotrophoblaste extravilleux invasif
IVS	Chambre intervilleuse
JNK	*C-jun-N-terminal kinase*
LH	Hormone luténisante
MAPK	Protéine MAP kinase

Mn	Manganèse
NQO2	Quinone réductase 2
$\bullet O_2^-$	Radical superoxyde
•OH	Radical hydroxyle
$ONOO^-$	Anion peroxynitrite
p-evCTB	Cytotrophoblaste extravilleux prolifératif
pGH	Hormone de croissance placentaire
PI3K	Phosphatidyl inositol 3-OH-kinase
PL-II	Lactogène placentaire-II
PL-Iv	Variante de la lactogène placentaire I
PLP-C	Protéine C prolactine-libre
PRL	Prolactine
RCIU	Restriction de croissance intra-utérine
RCPG	Récepteur couplé aux protéines G
RNS	Espèces réactives de l'azote
ROR	*Retinoic acid receptor-related orphan*
ROS	Espèce réactive oxygénée
RT-qPCR	Transcription inverse-Réaction en chaîne par polymérase quantitative
RZR	R*etinoid Z receptor*
SA	Artère spiralée utérine
SAPK	Protéine kinase activée par le stress
sEng	Endogline soluble
sFlt-1	Récepteur soluble de VEGF-1

siRNA	petit ARN interférant
Smac/DIABLO	*Second mitochondrial-derived activator of caspase/ direct inhibitor of apoptosis protein-binding protein with a low pI*
SOD	Superoxyde dismutase
STB	Syncytiotrophoblaste
STBM	Microparticule du syncytiotrophoblaste
TGF-α	Facteur de croissance transformant-alpha
TGF-β	Facteur de croissance transformant-beta
TNF-α	Facteur de nécrose tumorale-alpha
TM	Tunique musculaire
UV	Veine utérine
vCTB	Cytotrophoblaste villeux
vTB	Trophoblaste villeux
VEGF	Facteur de croissance vasculaire endothéliale
XDH	Xanthine déshydrogénase
XO	Xanthine oxydase
Zn	Zinc

INTRODUCTION

La prééclampsie est une importante complication obstétricale caractérisée par une hypertension et une protéinurie chez la mère qui se développe après la vingtième semaine de la grossesse (Sibai *et al.*, 2005). La prééclampsie est la plus grande cause de mortalité et morbidité maternelle et périnatale (WHO, 2005). La cause de la prééclampsie demeure inconnue, mais elle serait d'origine placentaire et est associée avec des altérations trophoblastiques (Redman *et al.*, 2009a). Les processus pathologiques conduisant à l'apparition du syndrome maternel impliquent un accroissement de l'apoptose mitochondriale du syncytiotrophoblaste, causée par des niveaux anormalement élevés de stress oxydatif (Hung *et al.*, 2006a, Redman *et al.*, 2009a). Des auteurs ont suggéré l'utilisation de la mélatonine, un puissant antioxydant, pour traiter la prééclampsie (Milczarek *et al.*, 2010, Okatani *et al.*, 2001a, Wakatsuki *et al.*, 2001) alors que la présence ou le rôle de la mélatonine dans le placenta provenant de grossesses normales ou compliquées par la prééclampsie demeurent peu étudiés.

Le placenta est un organe multifonctionnel indispensable au bon déroulement de la grossesse et de la croissance fœtale. La placentation de type hémomonochoriale accompagnée d'une intense activité endocrine est propre à l'espèce humaine (Malassine *et al.*, 2003). La villosité choriale, formée par le trophoblaste, est au cœur de cette particularité (Alsat *et al.*, 1999). Le trophoblaste humain se développe en deux voies, celle du trophoblaste villeux ou du trophoblaste extra-villeux. Le trophoblaste villeux est formé des cytotrophoblastes villeux (vCTB, cellules mononucléées) qui vont se fusionner et se différencier en syncytiotrophoblaste (STB, cellule multinucléée) (Aplin, 1991). Le syncytiotrophoblaste assure les échanges gazeux et de nutriments entre la mère et le fœtus et est le site de synthèse des hormones de la grossesse. Le trophoblaste villeux est un tissu ayant un taux de renouvellement élevé et le maintien de son homéostasie est essentiel au bon déroulement de la grossesse et de la croissance fœtale.

La mélatonine est une hormone au spectre d'activité biologique très vaste. Elle contrôle les rythmes biologiques, possède une puissante action antioxydante, régule la sécrétion de plusieurs hormones et facteurs de croissance, stimule la différenciation et inhibe l'invasion et la prolifération de différentes cellules (Claustrat *et al.*, 2005, Ekmekcioglu, 2006). Cette hormone est produite de façon rythmique, durant la période d'obscurité, dans la glande pinéale par l'activité séquentielle des enzymes aralkylamine N-acétyltransférase (AANAT) et hydroxyindole O-méthyltransférase (HIOMT) (Wurtman *et al.*, 1968). Il y a de plus en plus de preuves de sa synthèse dans différents tissus extra-pinéaux, notamment par le tractus gastro-intestinal, dont la

production est plus importante que celle de la glande pinéale (Huether, 1993). La présence de grandes quantités de mélatonine dans le placenta humain et de rat a été démontrée (Nakazawa *et al.*, 1999). Ces taux élevés, même en période diurne, ne montrent pas de variations circadiennes, suggérant une production extra-pinéale. Chez la femme enceinte, la production de mélatonine augmente significativement tout au long de la grossesse pour atteindre un maximum au troisième trimestre (Kivela, 1991, Nakamura *et al.*, 2001). La cause physiologique et la source de cette augmentation de mélatonine dans le sang maternel demeurent inconnues. Ces données suggèrent que la mélatonine pourrait être produite *de novo* par le placenta humain.

La mélatonine transmet son signal via l'activation des récepteurs membranaires MT1 et MT2, des récepteurs couplés aux protéines G, et par la famille des récepteurs nucléaires de type RZR/ROR (Reppert *et al.*, 1995, Reppert *et al.*, 1994, Smirnov, 2001). Il a été démontré que la mélatonine, par son importante action antioxydante, participe à la promotion de la survie cellulaire en inhibant l'apoptose mitochondriale, entre autres via l'activation de ses récepteurs MT1 et MT2 (Hardeland *et al.*, 2009, Rodriguez *et al.*, 2004, Tan *et al.*, 1999, Tan *et al.*, 1998, Tomas-Zapico *et al.*, 2005). En outre, la mélatonine traverse facilement, et sans subir de biotransformation, la barrière placentaire pour entrer dans la circulation fœtale (Okatani *et al.*, 1998). Par contre, la présence de récepteurs de la mélatonine dans le placenta humain demeure peu étudiée. Mes travaux de maîtrise ont mis en évidence la présence des récepteurs MT1, MT2 et RORα dans les lignées cellulaires JEG-3 et BeWo, des modèles *in vitro* du trophoblaste humain (Lanoix *et al.*, 2006). Ces données suggèrent que le trophoblaste humain exprime les récepteurs de la mélatonine et que cette hormone pourrait jouer un rôle bénéfique dans la survie du trophoblaste villeux.

La prééclampsie est une complication de la grossesse qui touche 3 à 15% des grossesses dans les pays développés. Elle est responsable d'une lourde morbidité maternelle (15-46%) et périnatale (>65% des mortalités fœtales) en plus d'être une cause importante de restrictions de croissance intra-utérine (RCIU) (10-15% des prééclampsies) et d'accouchements prématurés (15% des prééclampsies) (National Institute for Clinical Excellence, 2001, National Instiute for Clinical Excellence, 2004, Sibai *et al.*, 2005). Le développement de la prééclampsie est étroitement lié à celui du placenta. Il implique un défaut d'invasion du trophoblaste extravilleux et un dysfonctionnement généralisé du syncytiotrophoblaste. Ce dernier est notamment dû à une augmentation anormale des niveaux d'apoptose mitochondriale, causée par un état d'hypoxie/réoxygénation (Hung *et al.*, 2006a, Redman *et al.*, 2009a). Les taux sanguins maternels de mélatonine diminuent dans les cas de grossesses avec prééclampsie (Nakamura

et al., 2001). Ces données suggèrent qu'une altération de la signalisation mélatoninergique (récepteurs) et/ou de la production de la mélatonine par le trophoblaste villeux pourrait prendre part à la pathogénèse de la prééclampsie. À ce jour, le rôle de cette hormone dans un mécanisme conduisant à la pathogénèse de cette complication obstétricale n'a jamais été étudié.

Compte tenu des éléments de la littérature cités ci-haut, l'hypothèse de ce projet doctoral est que la mélatonine joue un rôle protecteur dans le maintien de la survie du trophoblaste villeux, essentiel au bon déroulement de la grossesse, et qu'une altération de la signalisation ou de la production placentaire de mélatonine est présente chez la grossesse compliquée par la prééclampsie et peut ainsi participer à sa pathogénèse. Ce projet comporte trois volets : 1) déterminer la production de mélatonine et l'expression de ses récepteurs chez le placenta humain à terme; 2) *in vitro,* déterminer la capacité de la mélatonine à prévenir l'apoptose mitochondriale du syncytiotrophoblaste induite par de l'hypoxie/réoxygénation; 3) caractériser la production de mélatonine et l'expression de ses récepteurs entre des placentas de grossesses normotensives et compliquées par la prééclampsie.

La première partie de cette thèse comporte une synthèse de la littérature présentant l'état des connaissances sur le placenta, la mélatonine et la prééclampsie. La deuxième partie est composée de cinq manuscrits rédigés à partir des résultats obtenus dans le cadre de ce projet de doctorat. Quatre articles ont été publiés dans les revues scientifiques *Journal of Pineal Research* en 2008, *Placenta* en 2012, *Molecular Biotechnology* en 2012 et *Journal of Pineal Reasarch* en 2012. Enfin, un dernier article a été soumis à *Molecular and Cellular Endocrinology*. Cette thèse comporte également une discussion générale ainsi que la contribution de ces travaux à l'avancement des connaissances de la mélatonine placentaire et de son importance pour le bon déroulement de la grossesse. Cette thèse comporte deux annexes. La première annexe présente des résultats préliminaires, et les méthodes associées, caractérisant l'expression des récepteurs de la mélatonine dans le placenta provenant du premier trimestre de la grossesse. La deuxième annexe comporte trois publications à titre de premier auteur, une lettre à l'éditeur publiée dans la revue *Placenta* en 2010, un article de revue publié dans la revue *Molecular and Cellular Endocrinology* en 2012 et un chapitre de livre publié en 2012 dans *Pregnancy Disorders and Perinatal Outcomes.*

Effet protecteur de la mélatonine sur l'homéostasie du trophoblaste villeux : implications dans la grossesse normale et compliquée par la prééclampsie

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Synthèse

LE PLACENTA HUMAIN

Le placenta est un organe multifonctionnel (production hormonale, échanges mère-fœtus, immunomodulation) jouant un rôle essentiel dans le déroulement de la grossesse et dans la croissance fœtale chez les mammifères. Bien que ses fonctions soient essentielles, le placenta est un organe dont le fonctionnement est toujours peu connu. Chez les mammifères, le placenta est l'organe dont la structure et le fonctionnement divergent le plus d'une espèce à l'autre en raison des différences de besoins nutritionnels, du nombre de rejetons et de la durée de la période de gestation. Une importante activité endocrine et une placentation de type hémomonochoriale sont propres à l'humain (Malassine *et al.*, 2003). La villosité choriale (ou villosité placentaire) constitue l'unité structurale et fonctionnelle du placenta humain (Alsat *et al.*, 1999). Elle baigne directement dans le sang maternel, apporté par les artères utérines, et elle est soit ancrée dans l'utérus maternel et appelée villosité crampon ou soit dans la chambre intervilleuse et appelée villosité flottante (**Fig.1**).

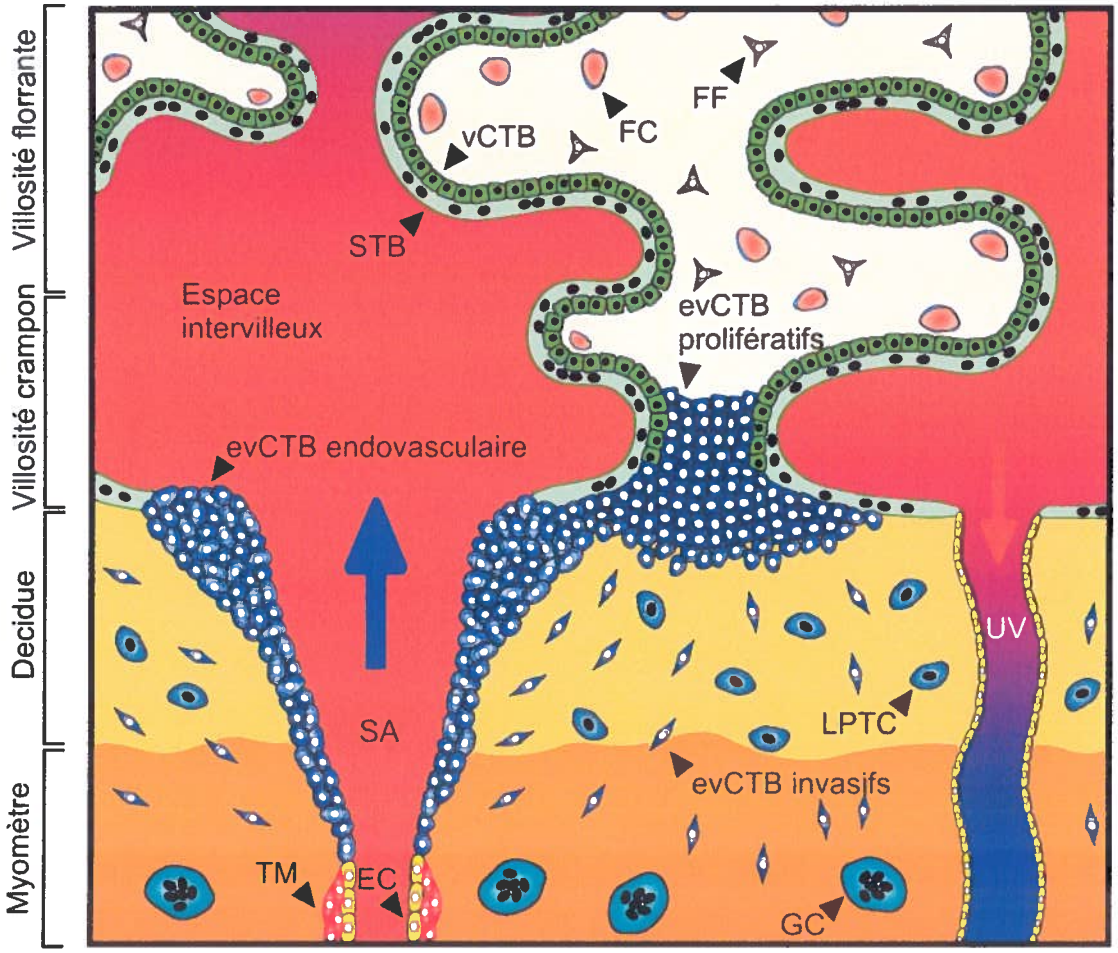

Figure 1 : Représentation schématique de la villosité choriale à l'interface foeto-maternel. La villosité flottante est en contact directe avec le sang maternel situé dans l'espace intervilleux. La villosité flottante est composée des cytotrophoblastes villeux (vCTB) et du syncytiotrophoblaste (STB). La villosité crampon est composée d'une population hétérogène de cytotrophoblastes extravilleux (evCTB) et des cellules géantes trophoblastiques (GC). EC : cellule endothéliale; FC : capillaire fœtal; FF : fibroblaste fœtal; LPTC : grande cellule polygonale trophoblastique; SA : artère spiralée utérine; TM : tunique musculaire; UV : veine utérine. Modifié de Lanoix *et al.* (Lanoix *et al.*, 2012a).

Développement du placenta humain

Dans la grossesse normale, la formation des villosités choriales s'initie 13 jours après la conception. Le centre des villosités choriales est composé du stroma fœtal et la couche épithéliale externe est formée par le trophoblaste. Les villosités crampons permettent l'implantation et la maintenance au début de la grossesse alors que les villosités flottantes contrôlent la croissance placentaire et les échanges transplacentaires. Le phénotype de trophoblaste présent dans les villosités flottantes sont le trophoblaste villeux alors que le trophoblaste extravilleux est localisé dans les villosités crampons (Benirschke *et al.*, 2006a) (**Fig. 2**).

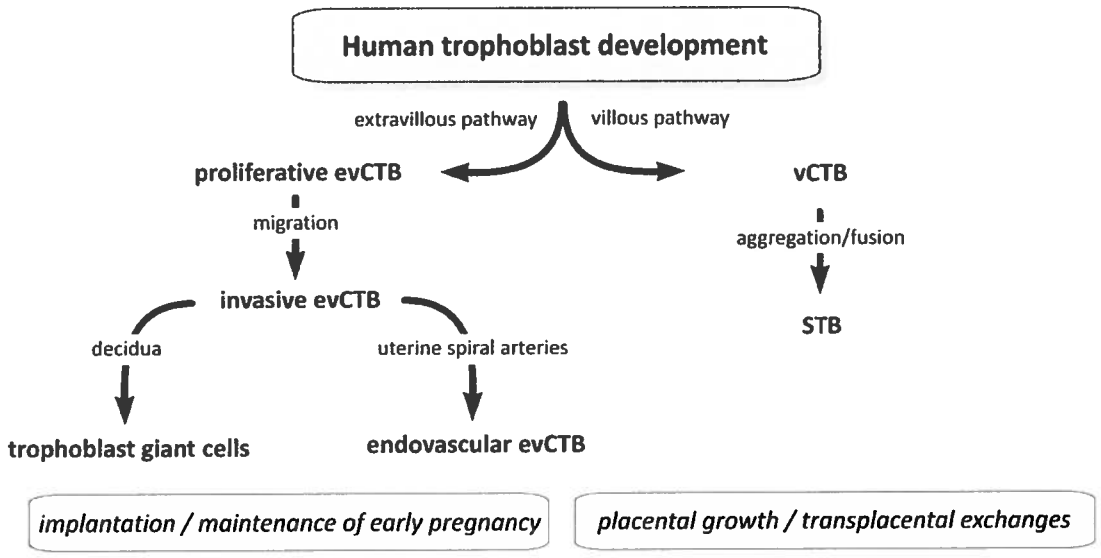

Figure 2 : Voies de différenciation du trophoblaste humain. evCTB : cytotrophoblaste extravilleux; STB : syncytiotrophoblaste; vCTB : cytotrophoblaste villeux. Tiré de (Vaillancourt *et al.*, 2009).

Migration et invasion du trophoblaste extravilleux

Le trophoblaste extravilleux est formé des cytotrophoblastes extravilleux, une population de cellules non polarisées au phénotype hétérogène (**Fig. 1**). La base des villosités crampons est formée par une colonne de cytotrophoblastes extravilleux. Les cellules composant la couche adjacente au stroma fœtal sont des cytotrophoblastes extravilleux prolifératifs. Les cellules de la couche distale de la colonne, sorties du cycle cellulaire, acquièrent un phénotype invasif. Les cytotrophoblastes extravilleux invasifs se propagent de la partie distale de la colonne jusqu'à la décidue et au myomètre (Aplin, 1991). Ces cytotrophoblastes très invasifs vont soit envahir le lit placentaire ou les artères spiralées utérines. Dans le lit placentaire, les cytotrophoblastes extravilleux invasifs vont se différencier en de grandes cellules trophoblastiques polygonales ou en cellules géantes trophoblastiques multinucléées. Les artères spiralées utérines seront envahies par les cytotrophoblastes extravilleux qui acquerront un phénotype endothélial, en remplaçant ainsi les cellules endothéliale des artères spiralées, et participeront à la dégradation de la tunique musculaire lisse pour enfin se différencier en cytotrophoblastes extravilleux endothéliaux (Kemp *et al.*, 2002, Pijnenborg *et al.*, 1983, Pijnenborg *et al.*, 1981, Winterhager *et al.*, 2000, Zybina *et al.*, 2004). Les artères spiralées sont ainsi remodelées; passant de petits vaisseaux fortement contractés à de larges vaisseaux atones (**Fig. 1**). Ce remodelage des

artères spiralées est essentiel pour permettre une bonne perfusion placentaire de manière à soutenir la croissance fœtale et le bien-être de la grossesse (Kaufmann *et al.*, 2003).

Syncytialisation

La différenciation du trophoblaste villeux est un processus biologique très complexe se produisant autant *in vivo* qu'*in vitro*. Malgré son rôle essentiel dans la grossesse et le développement fœtal, les mécanismes qui contrôlent la différenciation du trophoblaste villeux restent mal compris. Les premiers indices convaincants de la fusion trophoblastique en un syncytium, *in vivo*, ont été rapportés par Richart en 1961, grâce à des études d'incorporation de [^{3}H]-thymidine (Richart, 1961). Les connaissances sur la caractérisation de la différenciation du trophoblaste villeux ont été grandement améliorées depuis qu'il est possible d'isoler et de maintenir les cytotrophoblates villeux en culture primaire (Kliman *et al.*, 1986).

Kliman *et al.* (1986) ont développé une méthode pour isoler et purifier les cytotrophoblastes villeux à partir de placentas humains. Il s'agit de digestions enzymatiques séquentielles à l'aide de trypsine et de DNase suivies d'une purification sur gradient de Percoll discontinue (Kliman *et al.*, 1986). Ils ont démontré qu'*in vitro*, les cytotrophoblastes villeux fraîchement isolés vont fusionner et se différencier en syncytiotrophoblaste fonctionnel (Kliman *et al.*, 1986) (**Fig. 3**). Les modèles animaux ne sont pas appropriés pour les études portant sur la différenciation du trophoblaste villeux humain en outre parce qu'aucun type de placenta identifié à ce jour n'est comparable à l'humain et qu'aucun ne possède les mêmes types de cellules trophoblastiques que le placenta humain (Benirschke *et al.*, 2006c). Les cultures primaires de cytotrophoblastes villeux isolés de placentas humains sont donc un modèle de choix pour décrypter la différenciation du trophoblaste villeux. Dans ce modèle *in vitro*, la différenciation des cytotrophoblastes villeux en syncytiotrophoblaste se produit en quatre jours de culture (Kliman *et al.*, 1986) (**Fig. 3**). Ce processus est caractérisé par une différenciation morphologique et biochimique. La différenciation morphologique se définit par la fusion des cytotrophoblastes mononucléés en un syncytium adjacent, tandis que la différenciation biochimique se caractérise par la production d'hormones telles que l'hormone gonadotrophine chorionique humaine (hCG) et l'hormone lactogène placentaire (hPL) (Kliman *et al.*, 1986, Midgley *et al.*, 1963, Morrish *et al.*, 1987, Strauss *et al.*, 1992).

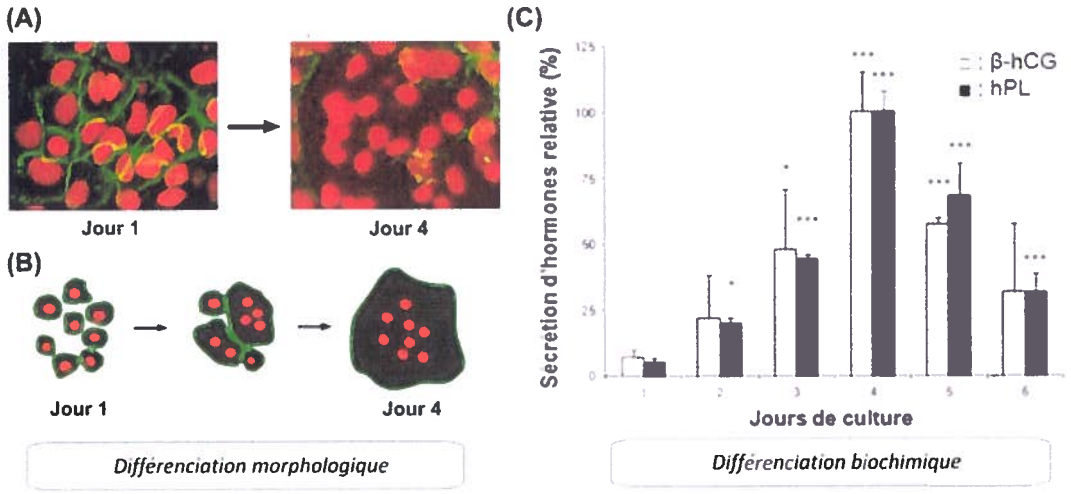

Figure 3 : Composantes de la différenciation du trophoblaste villeux. (A) Différenciation morphologique dans des cultures primaires de trophoblaste villeux de placenta humain à terme. Marquage de la desmoplakine et des noyaux par microscopie confocale après un et quatre jours de culture (400X). (B) Représentation schématisée de la différenciation morphologique *in vitro*. (C) Différenciation biochimique de cultures primaires de trophoblaste villeux de placenta humain à terme. Sécrétion relative de la sous-unité β de l'hormone gonadotrophine chorionique humain (β-hCG) et de l'hormone lactogène placentaire (hPL) de un à 6 jours de culture. Les données représentent la sécrétion relative ± SEM comparée à la sécrétion à quatre jours de culture (sécrétion maximale). $^{*}P < 0,05$; $^{***}P < 0,001$ comparé au premier jour de culture. Modifié de (Vaillancourt *et al.*, 2009).

L'évaluation de la différenciation morphologique du trophoblaste villeux est généralement effectuée par immunomarquage des desmoplakines et des noyaux (Douglas *et al.*, 1990). Au court de la fusion des cytotrophoblastes villeux, il y a une redistribution de la localisation des desmoplakines, une protéine associée au complexe jonctionnel des desmosomes (Douglas *et al.*, 1990). En effet, lorsque les frontières entre les cellules sont abolies pour former un syncytium, le marquage des desmoplakines disparait. Typiquement, *in vitro*, un syncytium est défini comme trois ou plusieurs noyaux dans un même cytoplasme sans qu'il y ait marquage des desmoplakines entre les noyaux (Douglas *et al.*, 1990). D'autres protéines de jonctions peuvent être utilisées pour évaluer la différenciation morphologique, dont la connexine 43, E-cadhérine et la syncytine (Alsat *et al.*, 1996, Frendo *et al.*, 2003a, Frendo *et al.*, 2003b).

La différenciation biochimique est généralement évaluée par la caractérisation de l'expression de l'ARNm (RT-qPCR) et de la sécrétion dans le surnageant de culture cellulaire (ELISA) de la hCG, la hPL et l'hormone de croissance placentaire (pGH) (Daoud *et al.*, 2006, Kliman *et al.*, 1986, Tarrade *et al.*, 2001). Autant la différenciation morphologique que

biochimique, qui peuvent être contrôlées indépendamment, sont nécessaires à l'analyse de la différenciation du cytotrophoblaste villeux en syncytiotrophoblaste.

In vitro, la présence de sérum est nécessaire pour une différenciation morphologique et biologique complète du cytotrophoblaste villeux. En effet, les cytotrophoblastes villeux isolés et maintenus dans des conditions sans sérum ne peuvent s'agréger ou fusionner et montre un faible degré de différenciation spontané (Morrish *et al.*, 1987). Des études ont démontré qu'*in vitro*, la différenciation des cytotrophoblastes villeux en syncytiotrophoblaste peut être induite ou inhibée par différents facteurs. Par exemple, les facteurs de croissance tels que le facteur de croissance épidermique (EGF), le facteur de croissance transformant-alpha (TGF-α), le facteur stimulant les colonies de granulocytes et macrophages (GM-CSF) et le facteur de croissance vasculaire endothéliale (VEGF), l'AMP cyclique (AMPc), les polypeptides ou les hormones stéroïdiennes, comme l'estrogène, la hCG et les glucocorticoïdes induisent la différenciation (Crocker *et al.*, 2001, L. Cronier *et al.*, 1998, L. Cronier *et al.*, 1994, L. Cronier *et al.*, 1999b, Garcia-Lloret *et al.*, 1994, Keryer *et al.*, 1998, Morrish *et al.*, 1987, Shi *et al.*, 1993, Yang *et al.*, 2003). En contraste, l'hypoxie, le facteur de croissance transformant-beta (TGF-β), le facteur de nécrose tumorale-alpha (TNF-α) et l'endotheline empêchent, *in vitro*, la formation du syncytium du cytotrophoblaste villeux et inhibent la sécrétion de hCG et hPL (Alsat *et al.*, 1996, L. Cronier *et al.*, 1999a, Leisser *et al.*, 2006, Morrish *et al.*, 1991).

La fusion des cytotrophoblastes villeux est un évènement clé de la formation du syncytiotrophoblaste. Plusieurs études ont examiné les mécanismes derrière la fusion des cytotrophoblastes villeux. En effet, les protéines impliquées dans les interactions cellule-cellule sont susceptibles d'être impliquées dans la fusion des cytotrophoblastes villeux. Par exemple, l'expression de la connexine 43, une protéine des jonctions communicantes, a été identifiée comme essentielle à la formation du syncytium (Laurent Cronier *et al.*, 2003, Frendo *et al.*, 2003a). D'autres études ont aussi démontré le rôle du rétrovirus endogène humain (HERV) comme étant une protéine d'enveloppe dans la fusion du cytotrophoblaste. La glycoprotéine fusogénique membranaire résultante de sa traduction a été nommée syncytine 1 et est exprimée dans tous les types de cellules trophoblastiques humaines (Malassine *et al.*, 2005). La transfection de la syncytine 1 dans des cellules non-trophoblastiques induit spontanément leur fusion alors que l'utilisation de petits ARN interférents (siRNA) codant pour HERV-W chez des vCTB inhibe la formation des STB et la sécrétion de hCG (Frendo *et al.*, 2003b). Une seconde glycoprotéine d'enveloppe impliquée dans la syncytialisation du trophoblaste et provenant du rétrovirus HERV-FRD a été identifiée récemment et se nomme syncytine 2 (Blaise *et al.*, 2003).

Cette protéine de fusion est exprimée dans les vCTB et dans les STB mais est absente dans les evCTB (Malassine *et al.*, 2007, Vargas *et al.*, 2012). La syncytine 2 diffère de la syncytine 1 parce qu'elle est seulement exprimée dans le vTB et qu'elle n'active pas le même récepteur. Le récepteur liant la syncytine 2 n'a toujours pas été identifié (Blaise *et al.*, 2003, Malassine *et al.*, 2007). D'autres molécules impliqués dans la fusion cellulaire ont été décrite, mais des études plus approfondie sont nécessaire pour déterminer exactement leur rôle dans la syncytialisation du trophoblaste (Adler *et al.*, 1995, L. Cronier *et al.*, 2002, Getsios *et al.*, 2003, Huovila *et al.*, 1996, Huppertz *et al.*, 2006, Kudo *et al.*, 2004, MacCalman *et al.*, 1996).

Homéostasie du trophoblaste villeux

Le trophoblaste villeux est un tissu avec un taux de régénération très rapide. Tout au long de la grossesse, le syncytiotrophoblaste est continuellement régénéré grâce à la fusion des cytotrophoblastes villeux, sous-jacents ayant un potentiel de cellule souche, en syncytiotrophoblaste (**Fig. 4**). Afin de maintenir leur homéostasie, les tissus à régénération rapide, telle que le trophoblaste villeux, doivent mourir pour être remplacés. En effet, le syncytiotrophoblaste entre en apoptose de manière à permettre sa régénération par les cytotrophoblastes villeux (Black *et al.*, 2004, Huppertz *et al.*, 1999, Yusuf *et al.*, 2002). *In vitro*, sans la fusion des cytotrophoblastes villeux, la durée de vie du syncytiotrophoblaste n'est que de quelques jours (Castellucci *et al.*, 1990, Crocker *et al.*, 2004). La différenciation des cytotrophoblastes villeux en syncytiotrophoblaste se produit en 96 heures, suivie par la dégénérescence du syncytiotrophoblaste (**Fig. 3**). Cette dégénérescence est le résultat d'une mort par apoptose. Les voies intrinsèques et extrinsèques de l'apoptose prennent part à ce processus (Heazell *et al.*, 2009). Dans le cadre de cette thèse, l'emphase sera mise sur la voie de l'apoptose intrinsèque.

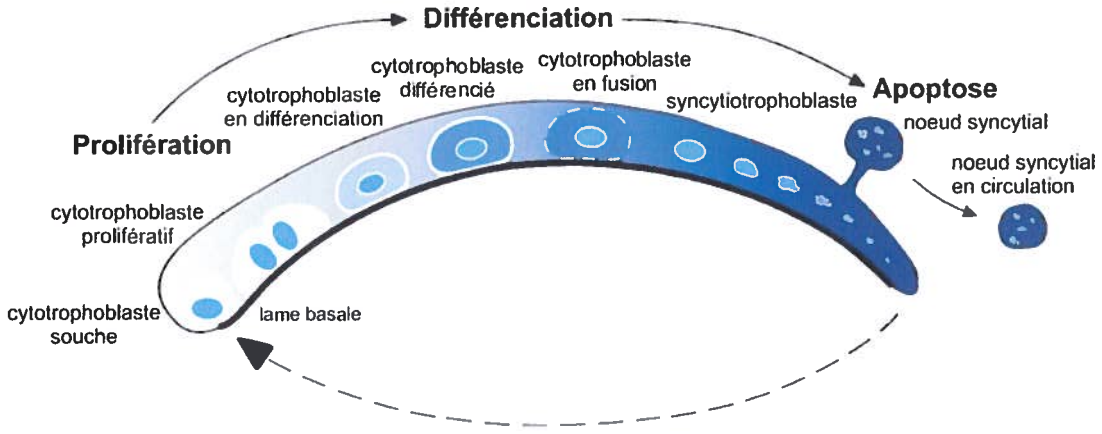

Figure 4 : Renouvellement du trophoblaste villeux. Le trophoblaste villeux est formé des cytotrophoblastes souches prolifératifs qui vont sortir du cycle cellulaire et se différencier en cytotrophoblastes villeux. Les cytotrophoblastes villeux mononucléés vont fusionner et se différencier en un syncytium, le syncytiotrophoblaste. La dégénérescence du syncytiotrophoblaste se produit par apoptose pour permettre sa régénération et donc de maintenir l'homéostasie du trophoblaste villeux. Modifié de Lanoix *et al.* (Lanoix *et al.*, 2012b).

Apoptose intrinsèque du trophoblaste villeux

La mitochondrie joue un rôle central dans la voie de l'apoptose intrinsèque qui est d'ailleurs également appelée apoptose mitochondriale (**Fig. 5**). Lors de dommages cellulaires, des signaux pro-apoptotiques parviennent à la mitochondrie, dont la voix de la protéine X associée (Bax)/ lymphome à cellules B-2 (Bcl-2), la protéine Smac/DIABLO (*second mitochondrial-derived activator of caspase/ direct inhibitor of apoptosis protein-binding protein with a low pI*) et le facteur induisant l'apoptose (AIF)/endonucléase G (EndoG). Les dommages à l'ADN déclenchent également la voie pro-apoptotique de p53 qui est activatrice de la voie de Bax/Bcl-2.

Dans la voie intrinsèque de l'apoptose, les molécules pro-apoptotiques de la famille de Bcl-2, telles que Bax et Bad, induisent une perméabilisation de la membrane mitochondriale (Deigner *et al.*, 2000). Il y a alors un relâchement de protéines de l'espace inter-membranaire dans le cytoplasme. Ces protéine pro-apoptotiques incluent le cytochrome c, AIF, Endo G et Smac/DIABLO (Namura *et al.*, 2001). Le cytochrome c permet ainsi la formation de l'apoptosome via sa liaison à APAF-1 (*apoptosis protease activating factor 1*) et à la pro-caspase-9 (Zamzami *et al.*, 2001). La caspase-9 est activée lors de la formation de l'apoptosome et permet à son tour l'activation de la caspase-3, entraînant la fragmentation et la

dégradation de l'ADN ainsi que la condensation de la chromatine (Lakhani *et al.*, 2006). Smac/DIABLO conduit directement à l'apoptose en inactivant la protéine inhibitrice de l'apoptose (IAP), inhibiteur direct de la caspase-3 (Orrenius *et al.*, 2007). AIF et Endo G vont transloquer au noyau, se lier à l'ADN et induire sa fragmentation ainsi que la condensation de la chromatine (Kroemer *et al.*, 2007). Les dommages à l'ADN vont également déclencher l'activation de p53, le principal régulateur de la survie de la cellule. Le facteur de transcription p53 va directement activer Bak, une protéine pro-apoptotique, et inhiber indirectement Bcl-2, un facteur anti-apoptotique, entraînant ainsi l'activation de la voie intrinsèque de l'apoptose (Mathai *et al.*, 2005). La mitochondrie est donc le principal régulateur de la cascade moléculaire conduisant à la mort cellulaire par la voie intrinsèque de l'apoptose (Mates *et al.*, 2008).

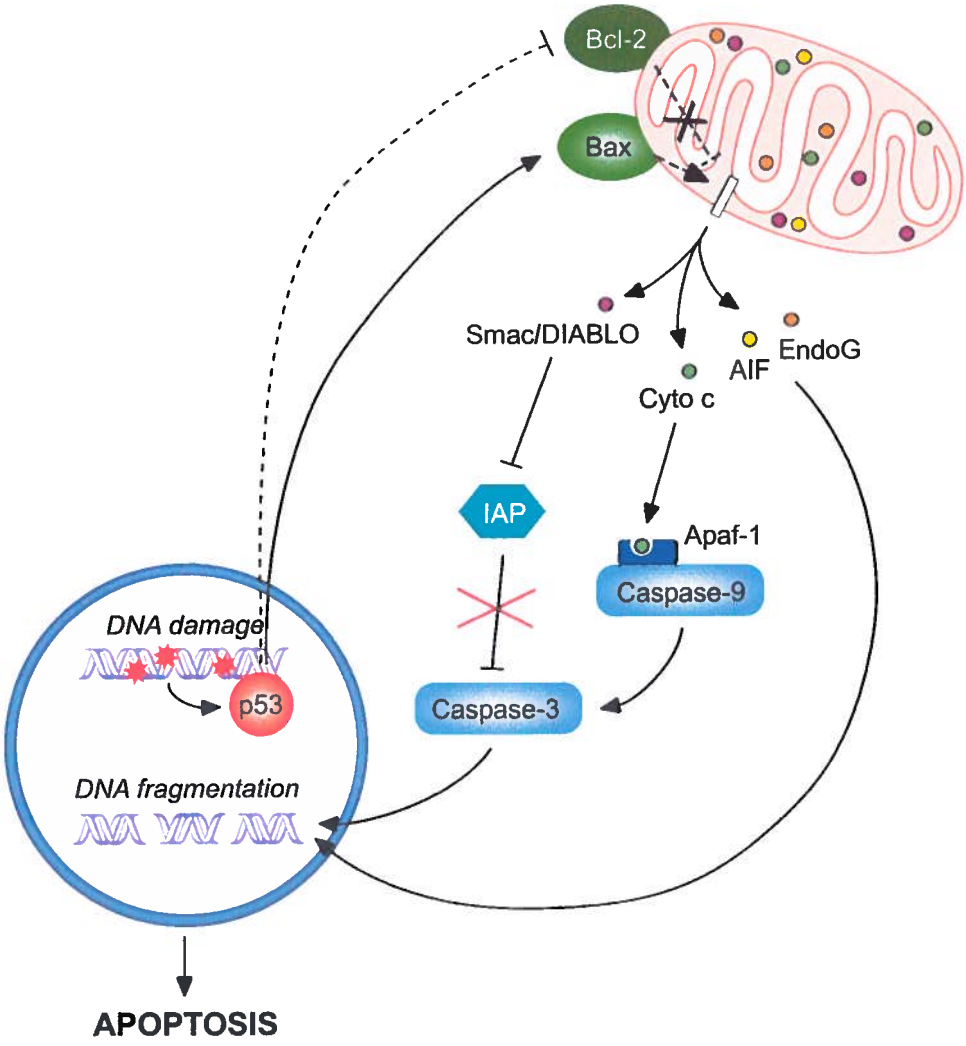

Figure 5 : Voie de l'apoptose intrinsèque. AIF : facteur induisant l'apoptose ; Apaf-1 : *apoptosis protease activating factor 1*; Bax : protéine X associée ; Bcl-2 : lymphome à cellules B-2; Cyto c : cytochrome c ; DNA : acide désoxyribonucléique ; EndoG : endonucléase G ; IAP : protéine inhibitrice de l'apoptose ; Smac/DIABLO : *second mitochondrial-derived activator of caspase/ direct inhibitor of apoptosis protein-binding protein with a low pI*. Tiré de Lanoix *et al.* (Lanoix *et al.*, 2012b).

Le maintien de l'homéostasie du trophoblaste villeux est un processus hautement régulé. Une altération des taux d'apoptose du syncytiotrophoblaste perturbe les fonctions placentaires et est impliquée dans de nombreuses complications de la grossesse. En effet, une augmentation des taux d'apoptose est observée dans les maladies du trophoblaste, telles que la môle hydatidiforme partielle ou complète et la formation de carcinome (Chiu *et al.*, 2001, Wong *et al.*, 1999). Lors d'un carcinome, il y a une augmentation de l'apoptose du syncytiotrophoblaste et de la prolifération des cytotrophoblastes villeux, significativement plus

élevée que dans les tissus normaux, entraînant la formation de tumeurs (Kale *et al.*, 2001). Une augmentation des taux d'apoptose du syncytiotrophoblaste est également observée chez certaines complications de la grossesse associée à un défaut placentaire, notamment la prééclampsie et la restriction de croissance intra-utérine (RCIU), tel qu'indiqué par une augmentation de l'expression de Smac/DIABLO, une diminution de Bcl-2 et une augmentation de la fragmentation de l'ADN (Allaire *et al.*, 2000, Heazell *et al.*, 2008, Ishihara *et al.*, 2002, Smith *et al.*, 1997, Tomas *et al.*, 2011).

LA MÉLATONINE

La mélatonine est présente de façon ubiquitaire dans la nature, identifiée dans la majorité des taxons, dont les bactéries, les eucaryotes unicellulaires, les végétaux, les invertébrés ainsi que les vertébrés (Pandi-Perumal *et al.*, 2006). Elle peut être produite de façon circadienne, mais ce n'est pas forcément le cas pour toutes les espèces (Hardeland *et al.*, 2003). Cette hormone représente, phylogénétiquement, un des plus anciens mécanismes de signal cellulaire connus.

Longtemps, il était admis que chez les mammifères, la mélatonine était uniquement produite par la glande pinéale. Ce n'est qu'à partir des années '70 que des études ont montré que cette hormone était aussi synthétisée de manière extra-pinéale par différents tissus et cellules, tels que le tractus gastro-intestinal, la peau, la rétine, les plaquettes, les lymphocytes, la moelle épinière et les testicules (Bubenik, 2002, Bubenik *et al.*, 1977, Bubenik *et al.*, 1974, Champier *et al.*, 1997, Conti *et al.*, 2000, Slominski *et al.*, 2008, Tijmes *et al.*, 1996, Tosini *et al.*, 1998). La régulation de la synthèse, le mode d'action et le rôle de la mélatonine extra-pinéale demeurent toutefois peu étudiés.

Synthèse de la mélatonine

La mélatonine est synthétisée à partir du L-tryptophane qui est d'abord transformé en sérotonine, la sérotonine est ensuite convertie en mélatonine par l'activité séquentielle de deux enzymes spécifiques : l'aralkylamine N-acétyltransférase (AANAT), qui acétyle la sérotonine en N-acétylsérotonine et l'hydroxyindole-O-méthyltransférase (HIOMT ou ASMT, acétylsérotonine-O-méthytransférase), qui convertit par méthylation la N-acétylsérotonine en 5-méthoxy N-acétyltrytamine (mélatonine) (**Fig. 6**) (Wurtman *et al.*, 1968). Chez l'humain, AANAT est

l'enzyme limitant la synthèse de la mélatonine cependant une controverse demeure (Klein *et al.*, 1997).

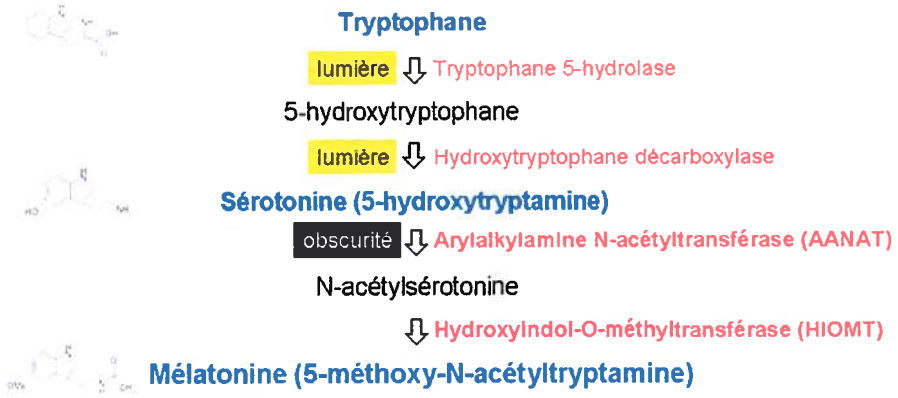

Figure 6 : Enzymes impliquées dans la synthèse de la mélatonine. Figure créée par D Lanoix.

La glande pinéale est considérée comme le site principal de synthèse de la mélatonine retrouvée dans la circulation sanguine. Au niveau de la glande pinéale, la production de cette hormone est directement contrôlée par la photopériode et la durée de sa synthèse est positivement corrélée à celle de la période d'obscurité (Pandi-Perumal *et al.*, 2006). La synthèse de mélatonine par les tissus périphériques n'est pas contrôlée par la photopériode et surpasse celle de la glande pinéale. Chez ces tissus, la concentration de mélatonine est donc supérieure à celle retrouvée dans le plasma, comme le tractus gastro-intestinal qui en produit 400 fois plus que les taux plasmatiques nocturnes (Huether, 1993).

Récepteurs de la mélatonine

La mélatonine exerce ses effets, entre autres, via l'activation de récepteurs à sept domaines transmembranaires de haute affinité, MT1 (mt1 ou Mel1a) et MT2 (Mel 1b) (Reppert *et al.*, 1995, Reppert *et al.*, 1994). Ces deux récepteurs font partie de la famille des récepteurs couplés aux protéines G (RCPG). Le site de liaison de la mélatonine, MT3, que l'on croyait être un troisième récepteur de la mélatonine, est en fait l'enzyme quinones réductases 2 (*dihydronicotinamide riboside : quinone reductase 2* (NQO2)) possédant un site de liaison pour la mélatonine (Mailliet *et al.*, 2004, Nosjean *et al.*, 2000). Des études ont démontré que le transcrit de NQO2 n'est pas exprimé dans le placenta humain (Jaiswal, 1994). Chez l'humain, la mélatonine semble également se lier aux récepteurs nucléaires de la famille RZR/ROR

(Smirnov, 2001). En outre, RORα (*retinoic acid receptor-related orphan*) et RZRβ (*retinoid Z receptor*) lient la mélatonine avec une haute affinité (Becker-Andre *et al.*, 1994, Giguere, 1999).

Chez l'humain, les récepteurs MT1 et MT2 sont présents dans le système nerveux central (hypothalamus, noyau suprachiasmatique, rétine, hypophyse) et en périphérie (lymphocytes, plaquettes, cellules de la granulosa, rein fœtal, artère coronaire, myomètre, adipocytes) (Dubocovich *et al.*, 2005, Ekmekcioglu, 2006). Le récepteur RORα est exprimé dans la plupart des tissus humains, tandis que RORβ est présent uniquement dans le cerveau, RORγ dans les muscles squelettiques, le thymus, le cœur, la prostate, les testicules, le pancréas et le foie, et RZRβ dans le cerveau, la rétine, la glande pinéale et la rate (Giguere, 1999, Smirnov, 2001). Enfin, l'expression des récepteurs de la mélatonine (MT1, MT2 et RORα) a été décrite, entre autres par mes travaux de maîtrise, dans les cellules de choriocarcinomes placentaires humains JAR, JEG-3 et BeWo, le tissu placentaire et les cellules trophoblastiques isolées de placentas de 1er trimestre de la grossesse (Iwasaki *et al.*, 2005, Lanoix *et al.*, 2006, Shiu *et al.*, 1999)

Rôles de la mélatonine

La mélatonine produite par la glande pinéale, secrétée dans la circulation sanguine, transmettra à toutes les structures centrales ou extra-pinéales, exprimant des récepteurs ou sites mélatoninergiques, l'information sur la photopériode, permettant une adaptation physiologique aux alternances jour/nuit ou aux saisons (Claustrat *et al.*, 2005). La mélatonine, une hormone très hydrophile et lipophile, est rapidement absorbée, traverse toutes les barrières morphophysiologiques (barrière hémato-encéphalique, placenta) et pénètre dans chaque partie de la cellule, dont le noyau (Okatani *et al.*, 1998, Pardridge *et al.*, 1980). Le spectre d'activité biologique de la mélatonine est très vaste. Elle contrôle les rythmes biologiques, régule le système immunitaire et possède une puissante action antioxydante et anti-inflammatoire (Claustrat *et al.*, 2005). De plus, la mélatonine a des propriétés hypotensives, notamment via l'activation de ses récepteurs MT1 et MT2, et a été suggérée comme étant le principal agent antihypertenseur du système cardiovasculaire (K-Laflamme *et al.*, 1998, Lew *et al.*, 1999, Regrigny *et al.*, 2001, Sewerynek, 2002). Enfin, plusieurs études proposent que la mélatonine joue un rôle protecteur dans le stade précoce et avancé de diverses maladies dans laquelle la pathogénèse implique des dommages induits par les radicaux libres, dont des complications de la grossesse telle que la prééclampsie (Allegra *et al.*, 2003, Milczarek *et al.*, 2010, Okatani *et al.*, 2001a, Reiter *et al.*, 2000, Reiter *et al.*, 2009, Sallinen *et al.*, 2007, Tomas-Zapico *et al.*, 2005, Wakatsuki *et al.*, 2001).

Promotion de la survie cellulaire

La survie de la cellule est compromise par l'apoptose mitochondriale, qui est notamment induite par le stress oxydatif. Une altération de l'équilibre pro-oxydant-antioxydant en faveur de la génération de radicaux libres induit l'apoptose mitochondriale (Allaire *et al.*, 2000, Ishihara *et al.*, 2002, Leung *et al.*, 2001). Les molécules antioxydantes, telles que les vitamines C (acide ascorbique) et E (α-tocophérol), le glutathion, les β-carotènes, le sélénium et la mélatonine agissent de manières non-enzymatiques en étant des chélateurs de radicaux libres (Bjelakovic *et al.*, 2011, Tan *et al.*, 2003). Plusieurs études ont démontré que la mélatonine est un puissant chélateur de radicaux libres, permettant d'inactiver le puissant radical hydroxyle (•OH) et plusieurs autres variétés d'espèces réactives oxygénées (ROS) (Bromme *et al.*, 2000, Fukutomi *et al.*, 2006, Matuszak *et al.*, 1997, Sofic *et al.*, 2005, Valko *et al.*, 2005). Plusieurs caractéristiques de la mélatonine font d'elle un excellent chélateur de radicaux libres permettant de diminuer l'apoptose des cellules. Son caractère lipophile et hydrophile lui permet de traverser facilement toutes les barrières morphophysiologiques et sa large distribution intracellulaire permet une grande protection contre les ROS et l'apoptose. La mélatonine est distribuée dans chacun des compartiments cellulaires, mais les taux sont particulièrement élevés au niveau de la mitochondrie et du noyau (Acuna-Castroviejo *et al.*, 2007, Menendez-Pelaez *et al.*, 1993). Lorsque la mélatonine chélate des radicaux libres, les métabolites qui en résulteront seront eux aussi d'excellents antioxydants (Hardeland *et al.*, 2009) (**Fig. 7**). Par exemple, lorsque la mélatonine chélate une molécule d'•OH, celle-ci est convertie en 3-hydroxymélatonine qui réagira avec un autre radical libre, générant ainsi une molécule de N^1-acétyl-N^2-formyl-5-méthoxykynuramine (AFMK), un puissant antioxydant. AFMK va chélater un autre ROS et être convertie en N^1-acétyl -5-méthoxykynuramine (AMK) (Tan *et al.*, 1999, Tan *et al.*, 1998). Ensuite, lorsque AFMK réagit avec une molécule de cations radicaux 2,2'-azino-bis (3-éthylbenzthiazoline-6- acide sulfonique) (ABTS) ou d'anions peroxynitrites ($ONOO^-$), une autre molécule antioxydante est produite (Guenther *et al.*, 2005, Than *et al.*, 2006). Cette cascade antioxydative contribue à faire de la mélatonine un puissant antioxydant permettant de protéger les cellules de l'apoptose.

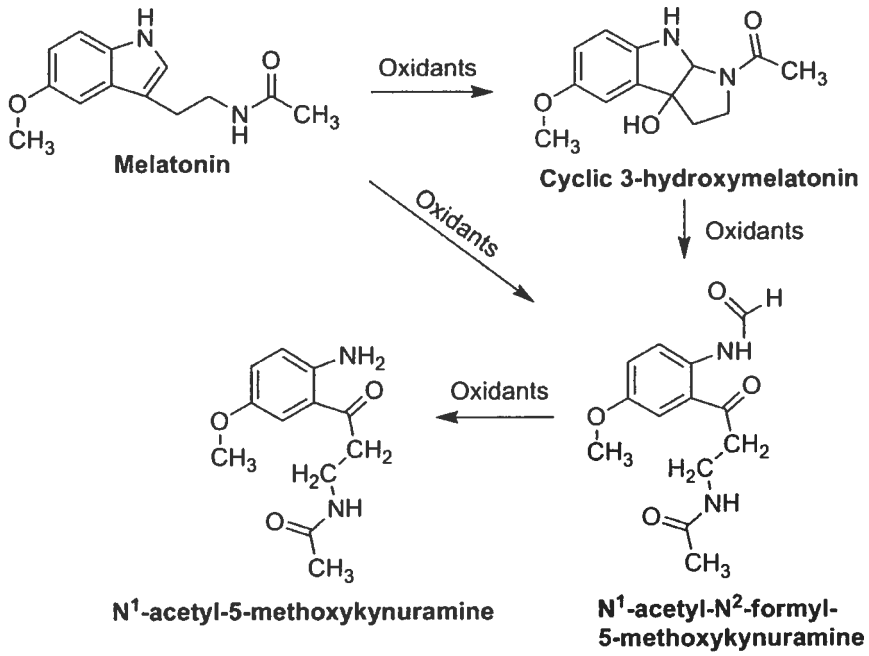

Figure 7 : Cascade antioxydative de la mélatonine. Tiré de Lanoix *et al.* (Lanoix *et al.*, 2012b).

Choi et ses collaborateurs (Choi *et al.*, 2011) ont démontré que la mélatonine prévient l'apoptose en augmentant l'expression des enzymes antioxydantes SOD1 et GPx de manières dépendantes de ses récepteurs MT1 et MT2. La mélatonine peut également inhiber l'apoptose en activant ses récepteurs (**Fig. 8**). Plusieurs études ont démontré que la mélatonine augmente l'expression et l'activité des enzymes antioxydantes, la superoxyde dismutase (SOD), la catalase (CAT) et la glutathion peroxidase (GPx), probablement via un mécanisme dépendant de ses récepteurs (Rodriguez *et al.*, 2004, Tomas-Zapico *et al.*, 2005) (**Fig. 8**). La capacité de la mélatonine à augmenter l'expression et l'activité de ces enzymes ainsi que sa capacité à chélater les radicaux libres font d'elle un des plus puissants antioxydants connus à ce jour (Bonnefont-Rousselot *et al.*, 2010, Jou *et al.*, 2010, Korkmaz *et al.*, 2009).

La mélatonine participe directement à la promotion de la survie cellulaire en inhibant l'apoptose de façons dépendantes de ses récepteurs. En effet, suite à sa liaison avec ses récepteurs membranaires MT1 et MT2, la mélatonine inhibe la phosphorylation des MAPK p38 et c-Jun N-terminal (JNK), bloquant l'activation de la protéine pro-apoptotique p53. L'inactivation de p53 diminue le ratio Bax/Bcl-2, inhibant la perte du potentiel membranaire mitochondrial.

Cette séquence d'évènements inhibe la relâche du cytochrome C et donc l'activation de la caspase-9 et de la caspase-3, permettant ainsi la survie de la cellule contre l'apoptose (Das *et al.*, 2008, Das *et al.*, 2010). La liaison de la mélatonine à ses récepteurs MT1 et MT2 inhibe également l'apoptose via l'activation de la voie de PI3K/AKT (Anhe *et al.*, 2004). L'activation de cette voie entraine la phosphorylation de Bad, une molécule pro-apoptotique (Datta *et al.*, 1997). Lorsque Bad est phosphorylé, il interagit avec la protéine chaperonne 14-3-3 pour inhiber sa translocation du cytoplasme vers la mitochondrie, promouvant ainsi la survie cellulaire (Choi *et al.*, 2008).

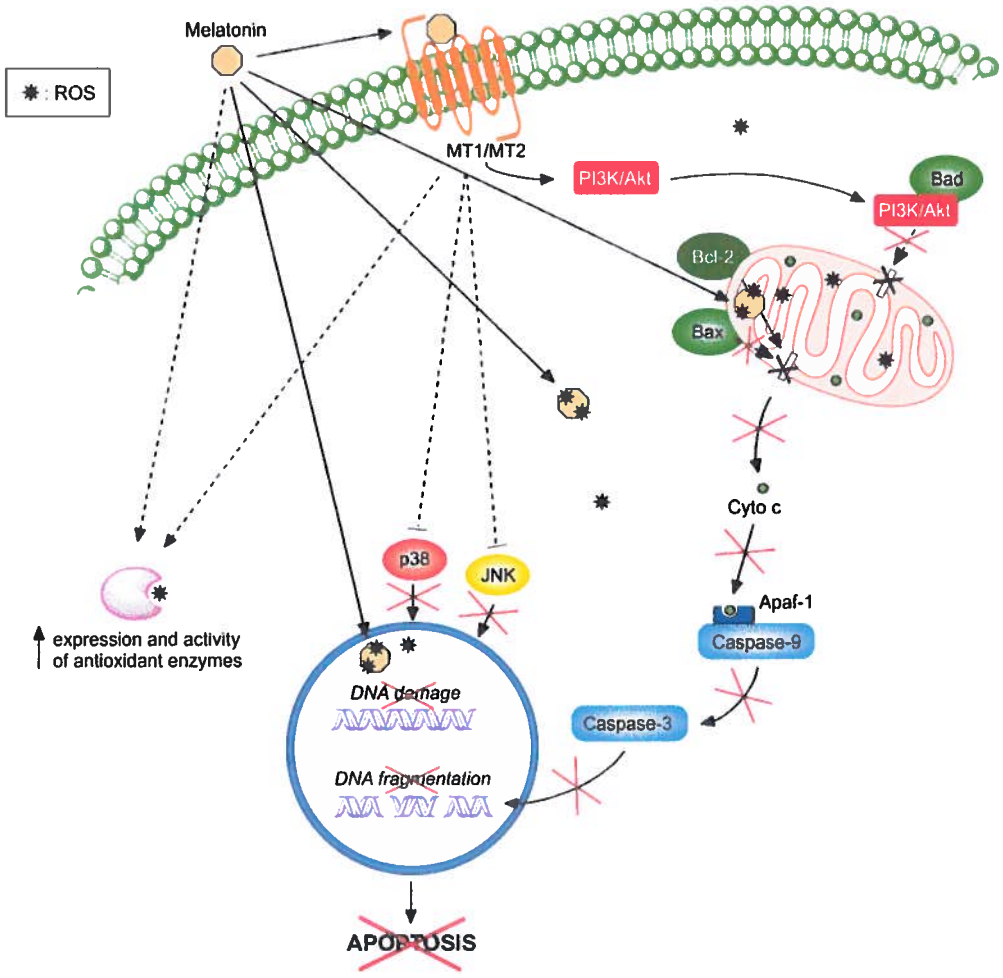

Figure 8 : Inhibition de l'apoptose intrinsèque par la mélatonine de façon dépendante et indépendante de ses récepteurs. Tiré de (Lanoix *et al.*, 2012b).

Mélatonine et la grossesse

La production de mélatonine augmente significativement tout au long de la grossesse pour atteindre un pic au 3^{e} trimestre. De plus, les taux sanguins de cette hormone en période nocturne et diurne sont plus élevés chez les femmes enceintes par rapport aux femmes non enceintes (Kivela, 1991, Nakamura *et al.*, 2001, Ogasawara *et al.*, 1991). La cause physiologique et la source de cette augmentation de mélatonine dans le sang maternel demeurent inconnues. Certains auteurs ont décrit des changements morphologiques et biochimiques (augmentation de l'activité des enzymes de synthèses) dans la glande pinéale chez les femmes enceintes par rapport aux femmes non enceintes (G. M. Lew, 1987) tandis que d'autres n'ont observé aucun effet de la grossesse sur la glande pinéale (Kivela, 1991). Ces travaux suggèrent que l'augmentation de mélatonine observée dans le sang des femmes enceintes pourrait être de source extra-pinéale et que le placenta pourrait en être la source. D'autre part, les hormones dont la production subit une augmentation aussi marquée durant la grossesse sont produites par le placenta comme la hPL (Evain-Brion *et al.*, 2003). La mélatonine traverse facilement, et sans subir de biotransformation, la barrière placentaire pour entrer dans la circulation fœtale via un mécanisme qui demeure inconnu (Okatani *et al.*, 1998). De plus, sa concentration dans le sang du cordon ombilical est significativement plus élevée que dans le sang maternel (Mitchell *et al.*, 1979). Ainsi, la mélatonine participerait à la régulation du développement fœtal. Les récepteurs MT1 et MT2 de la mélatonine ont été démontrés dans le cerveau et dans des tissus périphériques (par exemple, le rein, le foie et la rétine) dès la 18^{e} semaine de gestation chez le fœtus humain. En outre, le récepteur MT1 a été observé dans les cellules souches neuronales, suggérant qu'il jouerait un rôle dans le neurodéveloppement fœtal humain (Niles *et al.*, 2004). D'autre part, il a été démontré sur un modèle animal que la mélatonine maternelle est importante pour la croissance et pour la maturation sexuelle des rejetons (Bishnupuri *et al.*, 1999, Bishnupuri *et al.*, 2000, Bishnupuri *et al.*, 2001).

Le récepteur MT1 est exprimé tout au long de la grossesse dans le placenta des rates et son expression varie de manière spatiale et temporelle (Lee *et al.*, 2003). La même étude a démontré que l'expression du gène de la lactogène placentaire-II (PL-II, un équivalent de la hPL chez le rat) est modulée par le rythme circadien et que la variation observée est inverse à celle du gène du récepteur MT1. De plus, la 6-chloromélatonine (un agoniste sélectif et stable de la mélatonine) diminue l'expression des gènes de la PL-II, la variante de la lactogène placentaire I (PL-Iv) et de la protéine C prolactine-libre (PLP-C) dans le tissu placentaire de rates *in vitro* (Lee *et al.*, 1999). Nakazawa *et al.* ont montré la présence de mélatonine en grande quantité dans le

placenta humain et dans celui de rat même en période diurne (Nakazawa et al., 1999). Les taux de mélatonine dans le placenta humain à terme ne montrent pas la variation circadienne observée dans le sang maternel (Nakazawa *et al.*, 1999). Ces travaux suggèrent une production locale de mélatonine dans le placenta et que cette mélatonine endogène pourrait jouer un rôle différent de celle produite par la glande pinéale au cours de la grossesse. La mélatonine produite localement pourrait jouer un rôle paracrine/autocrine dans la régulation du développement et des fonctions endocrines placentaires. À ce jour, il existe peu d'études sur le rôle et le mode d'action de la mélatonine au niveau placentaire, tant chez l'humain que l'animal.

Altération de la mélatonine dans les grossesses pathologiques

Les taux sanguins maternels de mélatonine sont diminués dans les grossesses prééclamptiques ou avec RCIU par rapport aux grossesses normales (Nakamura *et al.*, 2001). De plus, les enfants nés de grossesses prééclamptiques ont un taux urinaire de 6-sulfatoxymélatonine (métabolite de la mélatonine) plus faible de 50% tandis que, dans le cas de RCIU, la baisse atteint 67% des niveaux mesurés chez les enfants nées de grossesses normales (Kennaway *et al.*, 2001). Une altération dans la fonction fœto-placentaire pourrait expliquer, en partie, la diminution de mélatonine observée dans le sérum maternel quoique le mécanisme précis demeure inconnu. D'autre part, une altération du transfert materno-placentaire et ombilico-placentaire de la mélatonine attribuable, en partie, à une modification du métabolisme de la mélatonine maternelle due aux variations de luminosité ont été décrits. Il a été démontré que le risque et la sévérité de la prééclampsie sont plus élevés chez les grossesses se déroulant durant les mois d'hiver dans l'hémisphère nord (période associée à une modification de la production de mélatonine) (Bider *et al.*, 1991, Magnu *et al.*, 2001, Phillips *et al.*, 2004). Il est important de souligner, que la lumière est un déclencheur des crises éclamptiques. Une approche préconisée pour prévenir ces crises est de maintenir les femmes en obscurité. À forte concentration, la mélatonine est reconnue pour avoir une action pharmacologique anticonvulsive chez l'humain (Molina-Carballo *et al.*, 1997). Ainsi, la diminution de mélatonine dans le sérum maternel observé dans les cas de prééclampsie pourrait être un des facteurs déclenchants des crises éclamptiques. De plus, la mélatonine inhibe la vasoconstriction des artères ombilicales humaines via une action dépendante de ses récepteurs (Okatani *et al.*, 2000a, Okatani *et al.*, 2000b, Okatani *et al.*, 2000c, Okatani *et al.*, 2001b). Ces données suggèrent que la mélatonine est impliquée dans l'étiologie de la prééclampsie. Par contre, si la diminution de la mélatonine plasmatique observée chez les femmes avec une

prééclampsie sévère et dans le cas de RCIU est la cause ou une conséquence de la maladie n'a jamais été démontré.

LA PRÉÉCLAMPSIE

La prééclampsie est une complication obstétrique, spécifique à l'humain qui se caractérise par une hypertension sévère associée à une protéinurie (et souvent un œdème) (Sibai *et al.*, 2005). Un chapitre de livre portant sur les désordres placentaires dans la prééclampsie est présenté en **Annexe 4** (Lanoix *et al.*, 2012a). L'éclampsie (du verbe grec *ekclampo*, jaillir/exploser) décrit un état convulsif paroxystique s'apparentant à l'épilepsie survenant surtout lors des derniers mois de la grossesse, pendant le travail ou post-partum qui évolue spontanément vers le coma et pouvant entraîner la mortalité maternelle et/ou fœtale (ACOG., 2002). La prééclampsie, si elle n'est pas contrôlée, peut se compliquer en éclampsie, d'où l'origine de son nom.

Épidémiologie et facteurs de risque

La prééclampsie est une des plus fréquentes complications de la grossesse ayant une incidence de 3 à 7% au Canada et jusqu'à 15 % dans les pays en développement, causant plus de 63 000 décès annuellement incluant les mères et leurs fœtus (WHO, 2003, WHO, 2005). Ce trouble est la cause la plus fréquente de décès, tant chez la mère que l'enfant, au cours de la grossesse et est responsable de 15 % des naissances prématurées dans les pays industrialisés. L'hypertension maternelle au cours de la grossesse est commune et affecte de 12 à 18 % de toutes les grossesses (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy, 2000). Environ 50 % des femmes présentant une hypertension gestationnelle développeront la prééclampsie (Chandiramani *et al.*, 2008). Les femmes enceintes nullipares représentent près de 75 % des cas de prééclampsie (Saftlas *et al.*, 2003, WHO, 2003). La sévérité et la fréquence de la prééclampsie sont, significativement plus élevées lorsqu'associé à certaines conditions, telles que la grossesse multipare (Chen *et al.*, 2009, Sibai *et al.*, 2009, Wen *et al.*, 2004), l'hypertension chronique (Marik, 2009, Sibai, 2002), le diabète (Howarth *et al.*, 2007, Peticca *et al.*, 2009), la thrombophilie pré-gestationnelle (Kahn *et al.*, 2009, Kupferminc, 2003) ainsi que des grossesses avec prééclampsie antérieure (Hernandez-Diaz *et al.*, 2009, Lykke *et al.*, 2009, McDonald *et al.*, 2009). Les facteurs de risque les plus communs sont présentés dans le **Tableau 1**.

Tableau 1 : Facteurs de risques de la prééclampsie. Modifié de (Lanoix *et al.*, 2012a).

Facteurs de risques maternels	Références
Grossesse multipare	**Chen *et al.*, 2009, Sibai *et al.*, 2000, Wen *et al.*, 2004**
Hypertension chronique	**Marik, 2009, Sibai, 2002**
Diabète pré-existant	**Howarth *et al.*, 2007, Peticca *et al.*, 2009**
Thrombophilie pré-gestationnelle	**Kahn *et al.*, 2009, Kupferminc, 2003**
Prééclampsie antérieure	**Hernandez-Diaz *et al.*, 2009, Lykke *et al.*, 2009, McDonald *et al.*, 2009**
Incisure proto-diastolique des artères utérines	**Rath *et al.*, 2009**
Âge maternel élevé ou faible	**Jahromi *et al.*, 2008, Najati *et al.***
Obésité et résistance à l'insuline	**Bodnar *et al.*, 2005a, Bodnar *et al.*, 2005b, Walsh, 2007**
Historique familial de prééclampsie	**Dekker *et al.*, 2001, Lie, 2007**
Susceptibilité génétique maternelle	**Ciarmela *et al.*, Nilsson *et al.*, 2004, Zusterzeel *et al.*, 2007**
Facteurs de risques paternels	Références
Primipaternité	**Campbell *et al.*, 1985, Skjaerven *et al.*, 2002**
Exposition au sperme limité	**Dekker, 2002, Robillard *et al.*, 1996**
Conception par techniques de procréation médicalement assistée	**Chen *et al.*, 2009, Wang *et al.*, 2002**

Le risque de prééclampsie est plus élevé chez les premières grossesses (Campbell *et al.*, 1985, Skjaerven *et al.*, 2002) et diminue lors d'expositions répétées au sperme avant la conception (Dekker, 2002, Robillard *et al.*, 1996). De plus, la conception par techniques de procréation médicalement assistée utilisant le sperme d'un donneur augmente le risque de prééclampsie (Salha *et al.*, 1999, Wang *et al.*, 2002). Ces études suggèrent un facteur immunologique et paternel dans la pathogénèse de la prééclampsie. Des données recueillies chez l'humain et des modèles animaux montrent que la déposition de sperme dans le tractus génital femelle provoque une réaction cellulaire similaire à une réponse inflammatoire classique; les

spermatozoïdes étant à l'origine de cette réaction. Ainsi, lorsque le tractus génital femelle est exposé de manière répétée au sperme avant la conception, une tolérance immunitaire de l'organisme maternel au sperme est induite. Enfin, lorsque la prééclampsie se développe avant la trentième semaine de gestation de la première grossesse, le taux de récurrence atteint 40 % chez les grossesses futures (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy, 2000).

Diagnostic

La prééclampsie est une maladie systémique hétérogène se développant après la vingtième semaine de la grossesse et antérieurement à 48 h post-partum (Sibai *et al.*, 2009). Les directives cliniques font la distinction entre la prééclampsie légère et sévère ainsi qu'entre la prééclampsie précoce (avant 34 semaines) et tardive (après 34 semaines) (Huppertz, 2008, von Dadelszen *et al.*, 2003). La distinction entre la prééclampsie précoce et tardive est le plus récent concept de classification. Les critères de diagnostic de la prééclampsie sont présentés au **Tableau 2** (American College of Obstetricians and Gynecologists, 2002). Généralement, la prééclampsie tardive présente des symptômes légers alors que la forme précoce est caractérisée par des symptômes sévères (Huppertz, 2008). Globalement, la prééclampsie survient tardivement dans plus de 80% des cas (Huppertz, 2008).

Tableau 2 : Critères de diagnostic de la prééclampsie.

	Tension artérielle	Protéinurie
Légère	≥ 140 mmHg/ ≥ 90 mmHg	> 3 g par jour
Sévère	≥ 160 mmHg/ ≥ 110 mmHg	> 3,5 g par jour
	Semaines de grossesse	
Précoce	< 34	
Tardive	≥ 34	

Pathogénèse de la prééclampsie

Dès le début de la décennie de 1960, la prééclampsie était connue sous le nom de « maladie des hypothèses » (Jeffcoate, 1966). De nos jours, la cause précise de la prééclampsie demeure toujours inconnue. Des efforts de recherches soutenues ont par contre conduit à des percées majeures dans la compréhension de la pathogénèse de cette maladie, probablement, multifactorielle. En se basant sur ces avancées, le concept d'un développement de la prééclampsie en 2 stades a été proposé en 1991 par le Dr Chris Redman (Redman, 1991).

Le premier stade survient avant l'apparition des signes cliniques (avant la 20^{e} semaine de la grossesse) et il est caractérisé par un défaut de placentation. Le second stade (après la 20^{e} semaine de la grossesse) est quant à lui caractérisé par un stress placentaire en réponse à la mauvaise placentation, ce qui va conduire au syndrome de la prééclampsie (hypertension, protéinurie et œdème). Bien que le modèle à plusieurs stades placentaires ait été révisé et défié, il demeure de loin la théorie la plus acceptée pour expliquer le développement de la prééclampsie (Huppertz, 2008, Redman *et al.*, 2005, Redman *et al.*, 2009a, Roberts *et al.*, 1999, Roberts *et al.*, 2009). Le Dr Redman et son collègue, le Dr Sargent, ont récemment révisé le modèle à 2 stades en modèle à 4 stades placentaires (Redman, 2011, Redman *et al.*, 2010). Une mauvaise adaptation immunitaire entre la mère et le fœtus serait à l'origine du défaut de placentation. Cette revue de littérature portera l'attention sur le modèle à 2 stades puisque c'est cette théorie qui est acceptée. Le modèle à 4 stades sera cependant discuté.

Rôle central du placenta

Le rôle central du placenta dans la pathogénèse de la prééclampsie est connu depuis plus d'un siècle (Eardley, 1909). Le développement de la prééclampsie dépend de la présence d'un placenta. De plus, le seul traitement définitif de la prééclampsie est l'élimination complète du placenta. Il a même été démontré que la présence d'un fœtus n'est pas requise pour que la prééclampsie se développe, telle que dans les grossesses molaires (Chun *et al.*, 1964). D'ailleurs, chez les cas de prééclampsie ou seulement le fœtus a été retiré, les symptômes maternels vont persister jusqu'à ce que le placenta soit retiré (Piering *et al.*, 1993, Shembrey *et al.*, 1995). De plus, des cas de prééclampsie post-partum ont été associés avec un retrait incomplet du placenta et les symptômes ont disparus suite à un curetage utérin (Matsuo *et al.*, 2007, Matthys *et al.*, 2004).

D'importantes modifications pathologiques sont observées dans les placentas de grossesses avec prééclampsie, notamment un flot sanguin utéro-placentaire insuffisant conduisant à un état d'ischémie-réperfusion et conséquemment au stress oxydatif placentaire. Chez les cas les plus sévères, cela peut même entraîner la formation d'infarctus placentaires. Les diverses altérations pathologiques du placenta dans la prééclampsie ne seront pas traité. Ils ont par contre été revus en détail par le Dr Benirschke et ses collègues en 2006 (Benirschke *et al.*, 2006b). Ces changements anatomopathologiques ne sont pas obligatoirement présents chez tous les cas de prééclampsie mais ils sont significativement plus fréquents (Moldenhauer *et al.*, 2003). D'autre part, ces lésions anatomiques ou histologiques ne sont pas utilisées comme critère de diagnostic, car ils ne sont pas spécifiques à la prééclampsie (Wynn, 1977).

Certains auteurs ont tentés d'établir une corrélation entre la sévérité de ces lésions et la sévérité du syndrome maternel, ce qui demeure controversé (Holzl *et al.*, 1974, Muller *et al.*, 1971, Salafia *et al.*, 1998, Schuhmann *et al.*, 1972). Bien qu'aucune lésion pathologique du placenta spécifique à la prééclampsie n'ait été démontrée, le placenta est l'élément essentiel au développement de cette complication de la grossesse. Enfin, une mauvaise implantation est un important facteur prédisposant au développement de la prééclampsie et le stress placentaire demeure la pierre angulaire au développement du syndrome maternel.

Stade 1 : mauvaise implantation

Un développement placentaire anormal, invasion et remodelage insuffisant des artères spiralées utérines par les cytotrophoblastes extravilleux, résultant en un apport sanguin réduit au placenta est appelé mauvaise implantation. La mauvaise implantation a été considérée comme étant la cause directe de la prééclampsie (Brosens *et al.*, 1972). Toutefois, comme une mauvaise implantation est également présente dans la grossesse normotensive avec des fœtus petits pour leur âge gestationnel, c'est donc probablement un important facteur prédisposant à la prééclampsie plutôt que sa cause (De Wolf *et al.*, 1980, Gerretsen *et al.*, 1981, Redman *et al.*, 1999).

Une réduction de l'invasion des artères spiralées utérines par les cytotrophoblaste extravilleux chez la grossesse avec prééclampsie a été identifiée pour la première fois en 1972 (Brosens *et al.*, 1972). En outre, une diminution du nombre de cytotrophoblastes extravilleux dans le lit placentaire ainsi qu'une diminution de la profondeur de l'invasion de ces cellules ont été démontrées dans la prééclampsie (Kadyrov *et al.*, 2006). Ces observations concordent avec un défaut de différenciation du phénotype prolifératif à invasif des cytotrophoblastes extravilleux dans la prééclampsie (**Fig. 9**) (Lim *et al.*, 1997, Redline *et al.*, 1995). De plus, dans la prééclampsie, une augmentation de l'apoptose des cytotrophoblastes extravilleux a été montrée dans le lit placentaire (DiFederico *et al.*, 1999, Genbacev *et al.*, 1999). En sommes, ces observations supportent l'invasion partielle du segment décidual des artères spiralées par les cytotrophoblastes extravilleux et l'importante diminution de l'invasion du myomètre (Meekins *et al.*, 1994). De plus, les cellules endothéliales des artères spiralées ne sont pas remplacées par des cytotrophoblastes extravilleux endovasculaire et les cellules des muscles lisses du segment du myomètre ne sont pas dégradées, résultant en un remodelage inadéquat des artères spiralées (Kaufmann *et al.*, 2003). Conséquemment, dans la prééclampsie, les artères spiralées utérines demeurent des vaisseaux petits, contractiles et de haute résistance, résultant en un flot sanguin utéro-placentaire artériel insuffisant et menant à la mauvaise implantation (**Fig. 9**)

(Harrington *et al.*, 1997, Papageorghiou *et al.*, 2002). La séquence d'évènements qui conduisent à la faible perfusion placentaire se produit avant la 20e semaine de la grossesse, avant l'apparence des signes cliniques. Il est donc difficile de déterminer quels sont les mécanismes impliqués dans le développement de la prééclampsie. Néanmoins, des facteurs impliqués dans l'invasion des cytotrophoblastes extravilleux, tels que la tension en oxygène, sont altérés dans la prééclampsie.

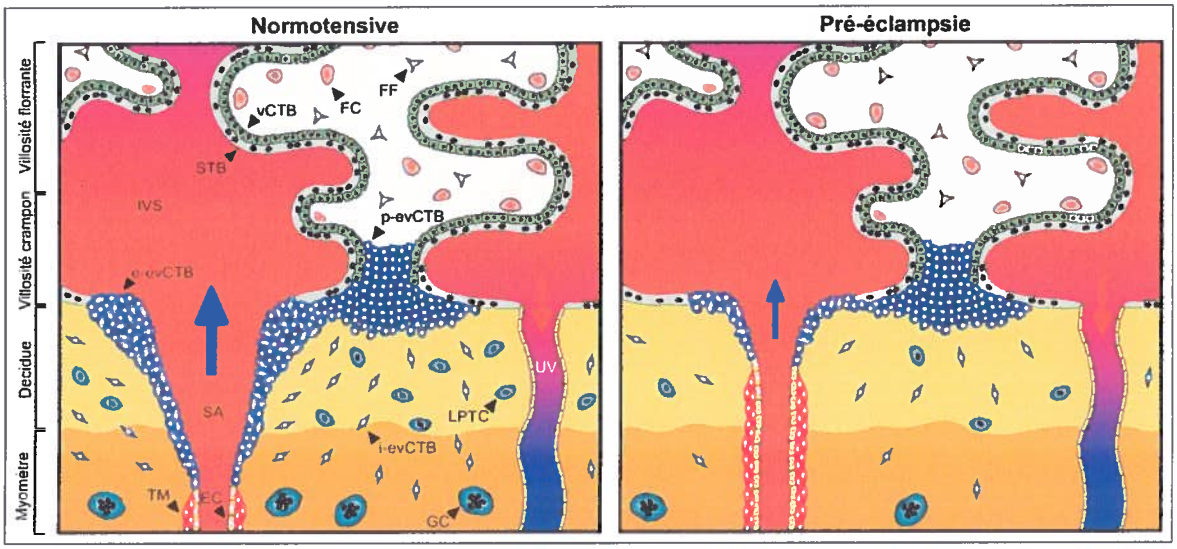

Figure 9 : Mauvaise implantation dans la grossesse compliquée par la prééclampsie. Modifié de (Lanoix *et al.*, 2012a).

Au début de la grossesse, avant la dixième semaine, le flot sanguin maternel au placenta est négligeable puisque les cytotrophoblastes extravilleux n'ont pas encore atteint et remodelé les artères spiralées (**Fig. 9**). Il en résulte donc un environnement hypoxique qui sera atténué lorsque le remodelage des artères spiralées sera effectué. De plus, la tension en oxygène est plus élevée dans les artères spiralées que dans le lit placentaire, créant un gradient croissant de la tension en oxygène qui semble agir comme un stimulus pour l'invasion des cytotrophoblastes extravilleux et leur différenciation en phénotype endovasculaire (Rodesch *et al.*, 1992). *In vitro*, une augmentation de la tension en oxygène stimule la différenciation des cytotrophoblastes extravilleux, de leur phénotype prolifératif à invasif (Genbacev *et al.*, 1997). De plus, à une tension en oxygène plus élevée, les cytotrophoblastes extravilleux expriment des molécules d'adhésion cellulaires similaires à celles retrouvées chez les cellules endothéliales vasculaires (Zhou *et al.*, 1998). Conséquemment, une diminution de la tension en oxygène altère la

différenciation et l'invasion des cytotrophoblastes extravilleux, reproduisant les évènements observés dans la prééclampsie (Genbacev *et al.*, 1996, Genbacev *et al.*, 1997, Zhou *et al.*, 1998). D'autre part, le facteur de transcription induit par l'hypoxie-1α (HIF-1 α) est exprimé dans des explants de placentas de premier trimestre de la grossesse cultivés sous une faible tension en oxygène alors que son expression diminue lorsque la tension en oxygène est augmentée (Caniggia *et al.*, 2000). L'expression et l'activité de HIF-1α sont fortement augmentées dans les placentas de prééclampsie (Rajakumar *et al.*, 2004, Rajakumar *et al.*, 2001). HIF-1α stimule l'expression du facteur de croissant transformant-β3 (TGF-β3), un inhibiteur de l'invasion chez les cytotrophoblastes extravilleux. Chez des explants de placentas, l'inhibition de l'expression de HIF-1α inhibe l'invasion et l'expression du TGF-β3 (Caniggia *et al.*, 1999). Dans l'ensemble, ces données indiquent que l'oxygène est un régulateur primordial de la différenciation et de l'invasion des cytotrophoblastes extravilleux. Par contre, il n'est toujours pas connu si l'hypoxie placentaire au début de la grossesse est la cause de la réduction de la différenciation et de l'invasion.

Stade 2 : Stress oxydatif placentaire, apoptose et syndrome maternel

Le stade final de la prééclampsie est un syndrome maternel caractérisé par l'apparition d'une hypertension et d'une protéinurie qui se résorbera après la délivrance du placenta à l'accouchement. Selon le modèle à deux stades placentaires –supporté par des données expérimentales chez l'humain – ces signes cliniques sont le résultat d'un stress inflammatoire systémique maternel en réponse à la sécrétion de plusieurs facteurs par le syncytiotrophoblaste. Le relâchement de ces facteurs est stimulé par l'apoptose du syncytiotrophoblaste engendrée par la mauvaise implantation (**Fig. 10**).

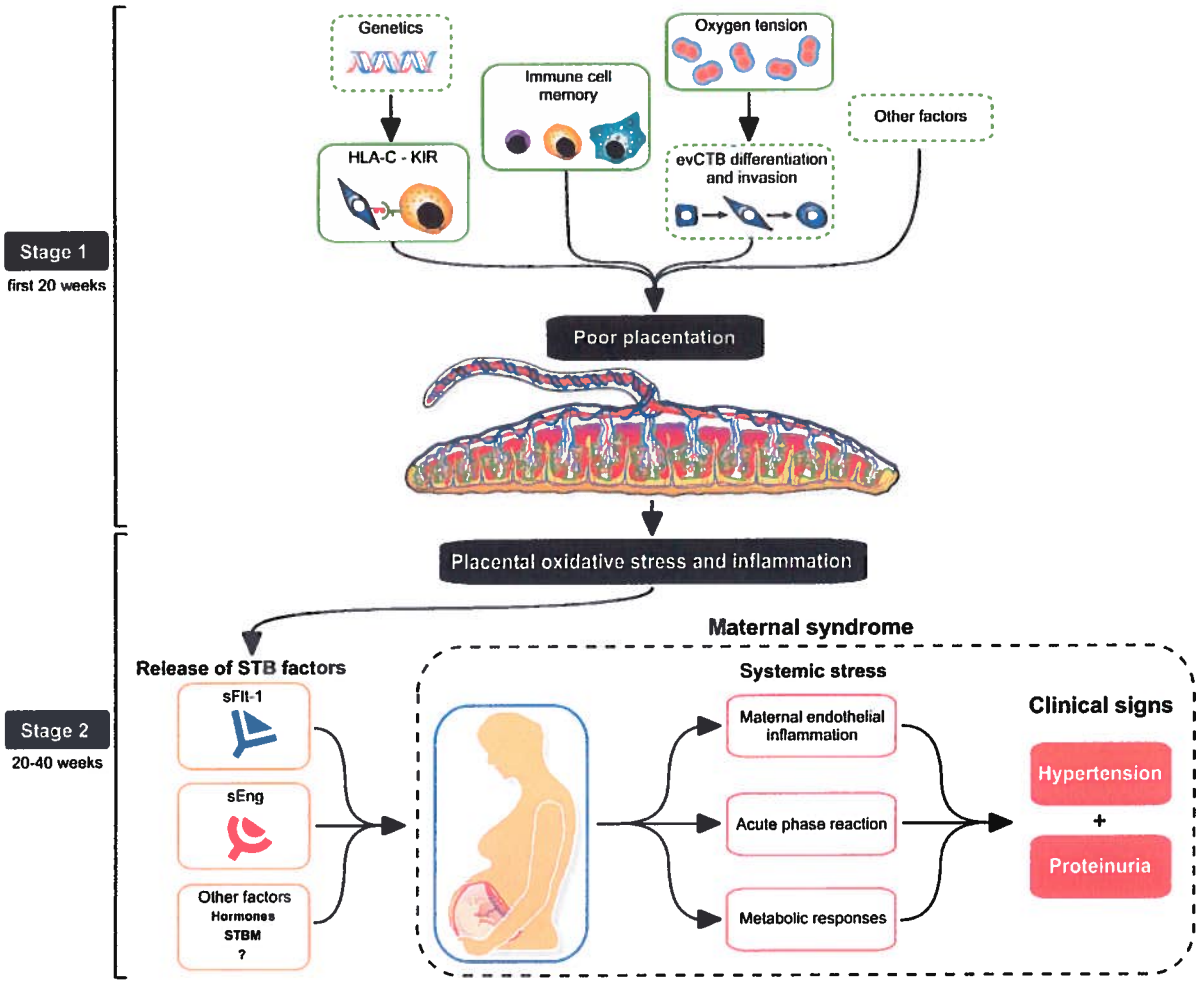

Figure 10 : Sommaire de la pathogénèse de la prééclampsie selon le modèle à deux stades placentaires. Tiré de (Lanoix *et al.*, 2012a).

Stress oxydatif placentaire

Au cours de la grossesse normale, une augmentation du stress oxydatif placentaire est observée alors que dans les cas de grossesses compliquées par la prééclampsie, cette augmentation est grandement amplifiée causant de nombreux dommages cellulaires menant à l'apoptose (Hubel, 1999, Hung *et al.*, Little *et al.*, 1999). De plus, de nombreux biomarqueurs du stress oxydatif placentaire lié à l'apoptose sont augmentés dans les cas de prééclampsie, tels que les taux de TNF-α, de 8-isoprostane, de radicaux superoxydes et de protéines carbonylées (Morikawa *et al.*, 1997, Shibata *et al.*, 2003, Sikkema *et al.*, 2001, Walsh *et al.*, 2000, Wang *et al.*, 1996b, Zusterzeel *et al.*, 2001). Conséquemment, le placenta est la principale source de stress oxydatif dans la prééclampsie et plusieurs études ont montré que la génération du stress

oxydatif placentaire est un événement clé de la pathogénèse puisqu'il induit l'apoptose (Burton *et al.*, 2004, Hubel, 1999, Redman *et al.*, 2000, Redman *et al.*, 2009a, Roberts *et al.*, 1999, Vanderlelie *et al.*, 2005).

Des études suggèrent que l'augmentation du stress oxydatif placentaire responsable de l'élévation de l'apoptose du syncytiotrophoblaste serait causée par le remodelage déficient des artères spiralées utérines (Burton *et al.*, 2001, Kaufmann *et al.*, 2003). Le remodelage déficient des artères utérines causerait ainsi une réduction du flux sanguin utéro-placentaire artériel et engendrerait donc un placenta chroniquement hypoxique (Burton *et al.*, 2001, Kaufmann *et al.*, 2003). Cependant, comme Hung et Burton ont rapporté, l'hypoxie placentaire chronique ne semble pas être l'inducteur du stress oxydatif et de l'apoptose dans les cas de grossesses compliquées par la prééclampsie (Hung *et al.*, 2006a). Chez le mouton, il est connu qu'une restriction chronique du flux sanguin chez les artères utérines est responsable d'une réduction du métabolisme placentaire ainsi que d'une diminution significative du poids du placenta et du fœtus (Lang *et al.*, 2000). Ce n'est cependant pas le cas dans la prééclampsie (Bloxam *et al.*, 1987, Xiong *et al.*, 2000). De plus, lors de grossesse en haute altitude, où l'apport en oxygène est limité au niveau du lit placentaire, le placenta ne montre pas de signes de dommages oxydatifs induisant l'apoptose (Espinoza *et al.*, 2001, Reshetnikova *et al.*, 1994). Finalement, le trophoblaste est tolérant à des taux réduits d'oxygène. Chez le premier trimestre, la concentration en oxygène au niveau du placenta est bien inférieure à celles du troisième trimestre jusqu'à ce qu'elle augmente à partir de la douzième semaine de la grossesse (Jauniaux *et al.*, 2000). Hung et Burton suggèrent que le stress oxydatif placentaire induisant un surcroît de l'apoptose (Hung *et al.*, 2002) durant la prééclampsie pourrait être induit par une hypoxie/réoxygénation (H/R) plutôt que par un état d'hypoxie seulement (Hung *et al.*, 2006a). Dans les cas de prééclampsie, le remodelage des artères spiralées utérines se fait seulement dans la région déciduale et elles demeurent pour la plupart vasoactives (**Fig. 9**). Le flux sanguin maternel entre donc dans l'espace intervilleux de manière pulsatile avec une haute pression, exposant le placenta à une tension d'oxygène arbitrairement oscillante (Jauniaux *et al.*, 1995, Jauniaux *et al.*, 1994). Ceci est d'ailleurs supporté par des études démontrant que l'H/R génère de hauts niveaux de radicaux libres, notamment les espèces réactives de l'azote (RNS) et les espèces réactives oxygénées (ROS) induisant l'apoptose, qui sont présentes dans les placentas de grossesses compliquées par la prééclampsie (Myatt *et al.*, 1996, Wang *et al.*, 2001). De plus, les explants placentaires de premier trimestre survivent très bien à de faibles niveaux d'oxygène, mais subiront un stress lorsque la tension en oxygène sera augmentée, induisant un

stress oxydatif et conséquemment de l'apoptose (Hung *et al.*, 2001, Hung *et al.*, 2002, Watson *et al.*, 1998).

Induction de l'apoptose mitochondriale par les espèces réactives oxygénées (ROS)

Dans les cas de prééclampsie, il y a une augmentation de l'apoptose mitochondriale des cellules placentaires induite par une surproduction de ROS. Les plus communément produits par le placenta sont les radicaux superoxydes ($\bullet O_2^-$) et le peroxyde d'hydrogène (H_2O_2) (Sikkema *et al.*, 2001). Ils sont principalement générés par deux sources intracellulaires, la voie de la xanthine déshydrogénase/xanthine oxydase (XDH/XO) et la chaîne de transport d'électrons mitochondrial. Dans les placentas de grossesses compliquées par la prééclampsie, une augmentation de l'expression de la XDH/XO ainsi que de l'activité de la XO est observée comparativement à ceux de grossesses normotensives (Many *et al.*, 2000) (Many *et al.*, 1996). De plus, la perfusion de placentas normaux avec de la XO induit des modifications d'expression de gènes reliés aux stress oxydatifs et à l'apoptose tels que décrits dans la prééclampsie (Centlow *et al.*, 2009). La chaine respiratoire mitochondriale est une autre principale source de production de ROS induisant l'apoptose dans la préeclampsie (Wang *et al.*, 1998). Durant la phosphorylation oxydative mitochondriale, les électrons sont transférés du nicotinamide adénine dinucléotide (NADPH) réduit ou de la flavine adénine dinucléotide ($FADH_2$) réduit à des molécules d'oxygènes (O_2) résultants en la synthèse d'ATP (Brown, 1992). Le processus de transfert d'électron génère des radicaux superoxydes (Jezek *et al.*, 2005, Pitkanen *et al.*, 1996). Dans les placentas normaux, les niveaux de radicaux superoxydes sont très étroitement régulés par la manganèse superoxyde dismutase (Mn-SOD), dans les mitochondries, et par la cuivre/zinc superoxyde dismutase (Cu/Zn-SOD), dans le cytoplasme (Weisiger *et al.*, 1973). La SOD catalyse la dismutation des radicaux superoxydes en peroxyde d'hydrogène et en molécules d'hydrogène (McCord *et al.*, 1971). Dans les cas de prééclampsie, une diminution de l'expression et de l'activité de la SOD a été démontrée, résultant en une augmentation des niveaux de radicaux superoxyde (Vanderlelie *et al.*, 2005, Y. Wang *et al.*, 1996a, Y. Wang *et al.*, 2001, Wiktor *et al.*, 1998). La conversion du peroxyde d'hydrogène en eau et en molécules d'oxygènes est catalysée par la catalase et la GPx (Hochstein *et al.*, 1968). Dans les placentas compliqués par une prééclampsie comparativement à ceux provenant de grossesses normotensives, aucune différence dans l'expression de la catalase n'à été trouvée, mais une augmentation de son activité a été démontrée. Cependant, l'expression et l'activité de la GPx sont diminuées dans les cas de prééclampsie, causant une moindre élimination du peroxyde d'hydrogène (Atamer *et al.*, 2005, Madazli *et al.*, 2002, Mistry *et al.*, Poranen *et al.*, 1996,

Vanderlelie *et al.*, 2005, Walsh *et al.*, 1993). En sommes, ces études montrent que la prééclampsie est caractérisée par une augmentation de la production de ROS au niveau du placenta ainsi que par une diminution des mécanismes de défense antioxydants placentaires qui sont les principaux inducteurs de l'apoptose mitochondriale.

Stress oxydatif et apoptose du trophoblaste villeux

Une augmentation de la production de ROS par le trophoblaste cause une vaste gamme d'effets cytotoxiques qui ultimement conduisent à l'apoptose du syncytiotrophoblaste. Ces effets cytotoxiques sont principalement des dommages aux protéines et aux lipides des cellulaires ainsi que l'activation de voies de signalisation. Les voies de signalisation induites par les ROS et les mécanismes conduisant à l'apoptose du syncytiotrophoblaste seront présentées.

Les ROS participent à la régulation de plusieurs processus cellulaire en agissant comme seconds messagers ou en modifiant des voies de signalisation qui vont altérer l'homéostasie de la cellule menant à l'apoptose mitochondriale. Dans le placenta, les principales voies de signalisations sont les MAPKs et le NF-κB (Cindrova-Davies, 2009). Les MAPKs sont une famille de protéines kinases impliquées dans plusieurs réponses physiologiques et mécanismes de régulation (Chen *et al.*, 2001). Les ROS sont des inducteurs connus de l'apoptose par les voies p38 et SAPK/JNK (Matsuzawa *et al.*, 2005). L'H/R active p38 et SAPK/JNK dans des explants de trophoblaste villeux, induisant la sécrétion de cytokines pro-inflammatoires et l'apoptose (Cindrova-Davies, 2009, Cindrova-Davies *et al.*, 2007). De plus, une augmentation de l'activation de p38 a été démontrée dans les placentas de grossesses compliquées par la prééclampsie (Shin *et al.*, 2009). NF-κB est un facteur de transcription dimérique. Une activation de NF-κB par les ROS va engendrer la transcription de gènes impliqués dans l'inflammation, la réponse au stress et l'apoptose, tels que HIF-1α (Jung *et al.*, 2003, Schreck *et al.*, 1992). L'H/R induit l'activation de NF-κB dans des explants de trophoblaste villeux, stimulant la sécrétion de cytokines pro-inflammatoires et l'apoptose (Cindrova-Davies *et al.*, 2007). Une augmentation de l'expression de NF-κB est observée dans les placentas de grossesses compliquées par la prééclampsie en comparaison à ceux de grossesses normotensives (Aban *et al.*, 2004). Les ROS activent donc des voies de signalisation dans le placenta qui vont altérer l'homéostasie cellulaire, induisant ainsi un stress inflammatoire placentaire et de l'apoptose.

L'effet le plus dévastateur des ROS est l'apoptose de la cellule. L'apoptose se produit dans le placenta de grossesse normale et augmente tout au long de la grossesse (Smith *et al.*, 1997b). Dans la grossesse normale, un niveau basal d'apoptose est responsable de la fusion

syncytiale ainsi que le renouvellement du syncytiotrophoblaste (Huppertz *et al.*, 1999, Levy *et al.*, 2000). Dans la prééclampsie, l'apoptose placentaire est significativement augmentée comparée à la grossesse normotensive (Allaire *et al.*, 2000). Des niveaux anormaux d'apoptose se produisent particulièrement dans le syncytiotrophoblaste (Allaire *et al.*, 2000, Ishihara *et al.*, 2002, Leung *et al.*, 2001). Conséquemment, le renouvellement du trophoblaste villeux est altéré dans la prééclampsie (Huppertz *et al.*, 2004). L'H/R induit l'apoptose du syncytiotrophoblaste *in vitro* ainsi que du stress oxydatif, reproduisant les changements se produisant dans la prééclampsie (Cindrova-Davies *et al.*, 2007, Hung *et al.*, 2002, Huppertz *et al.*, 2003). Il n'est actuellement pas connu si cette apoptose est un évènement pathologique primaire ou une manifestation secondaire. Le modèle à plusieurs étapes placentaires propose qu'il s'agisse d'un évènement secondaire se produisant suite à l'altération de l'invasion des cytotrophoblastes extravilleux (Redman, 1991, Redman *et al.*, 2005, Redman *et al.*, 2009a, Roberts *et al.*, 1999). Huppertz propose toutefois que l'apoptose du syncytiotrophoblaste soit la principale altération placentaire se produisant dans la prééclampsie (Huppertz, 2008). Néanmoins, les deux hypothèses supportent que le stress oxydatif perturbe l'architecture syncytiale en augmentant l'apoptose du syncytiotrophoblaste, stimulant la relâche de facteurs dans la circulation maternelle qui sont responsables de la réponse inflammatoire systémique et des signes cliniques (Huppertz, 2008, Redman *et al.*, 2009b).

Apoptose du trophoblaste villeux et syndrome maternel

Dans la prééclampsie, les facteurs relâchés par le syncytiotrophoblaste en réponse au stress oxydatif comprennent, l'activine-A, de l'ADN, le corticolibérine (CRH), la leptine et le TNF-α (Muttukrishna *et al.*, 1997, Lo *et al.*, 1999, Mise *et al.*, 1998, Perkins *et al.*, 1995, Y. Wang *et al.*, 1996b). Les plus étudiés sont les microparticules syncytiales (STBM), sFlt-1 et sEng (Maynard *et al.*, 2003, Redman *et al.*, 2008, Venkatesha *et al.*, 2006). *In* vitro, il a d'ailleurs été démontré que l'H/R, par l'apoptose du syncytiotrophoblaste, stimule la relâche d'ADN, de TNF-α, de STBM, de sFlt-1 et de sEng (Hung *et al.*, 2004, Huppertz *et al.*, 2003, Maynard *et al.*, 2003, Tjoa *et al.*, 2006, Venkatesha *et al.*, 2006). Ce cocktail de facteurs pro-inflammatoires syncytiaux va contribuer au développement de la réponse inflammatoire systémique maternelle.

Les caractéristiques cliniques de la prééclampsie sont l'apparition d'hypertension et de protéinurie qui se résorberont après l'accouchement. L'hypertension maternelle est le résultat d'une dysfonction endothéliale diffuse alors que la protéinurie est attribuée à une endothéliose glomérulaire (Gaber *et al.*, 1994, Roberts *et al.*, 1989). Une augmentation de l'expression et de l'activation de plusieurs marqueurs de dysfonction de l'endothélium est démontrée chez les

femmes ayant une grossesse compliquée par la prééclampsie (Clark *et al.*, 1992, Deng *et al.*, 1994, Friedman *et al.*, 1995, Lyall *et al.*, 1994, Minakami *et al.*, 1993, Roberts *et al.*, 1991). De plus, l'incubation de cellules endothéliales avec du sérum de femmes ayant une grossesse compliquée par la prééclampsie résulte en une dysfonction endothéliale, suggérant que les facteurs syncytiaux soient responsables de l'hypertension et la protéinurie (Venkatesha *et al.*, 2006).

En plus d'une inflammation de l'endothélium, la prééclampsie est caractérisée par une réaction de phase aiguë (Redman *et al.*, 2009a, Redman *et al.*, 2004). Il s'agit d'une réponse endocrine et métabolique complexe déclenchée par de nombreuses cytokines pro-inflammatoires principalement sécrétée par les macrophages et monocytes au site d'inflammation (Gabay *et al.*, 1999, Ruminy *et al.*, 2001). Ces cytokines vont stimuler ou inhiber la production de protéines de la phase aiguë par les hépatocytes qui vont notamment causer de la fièvre, de l'hypercoagulabilité ou encore une stimulation de l'inflammation.

Enfin, de nombreuses réponses métaboliques sont activées suite à l'inflammation systémique présente dans la prééclampsie; supportant la proposition que la prééclampsie n'est pas seulement une maladie endothéliale (Redman *et al.*, 2009a). Elles incluent principalement le métabolisme des lipides. L'hypertriglycéridémie, la résistance à l'insuline, une augmentation des taux circulant d'acides gras libres et de lipoprotéines de faible densité ainsi que la présence de lipoprotéines de faible densité oxydées caractérisent la prééclampsie (Hubel *et al.*, 1996, Ogura *et al.*, 2002, Qiu *et al.*, 2006).

L'implication de nombreuses composantes du réseau inflammatoire dans la pathogénèse de la prééclampsie indique une réponse plus systémique que seulement une dysfonction endothéliale.

Prévention et traitement de la prééclampsie

Le traitement le plus efficace pour la prééclampsie est l'expulsion du placenta. Ce n'est par contre pas toujours approprié, tel que dans les cas d'accouchements prématurés (American College of Obstetricians and Gynecologists, 2002, National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy, 2000). De manière générale, plus tard le bébé est né, le mieux c'est, sauf pour les cas de prééclampsie sévères où la croissance fœtale est compromise en raison de fonctions placentaires sévèrement altérées.

Plusieurs études cliniques ont rapporté l'efficacité de divers composés pour réduire la sévérité de la prééclampsie (Sibai *et al.*, 2005). Les méthodes les plus utilisées pour prévenir ou traiter la prééclampsie sont résumées au **Tableau 3**.

Tableau 3 : Stratégies préventives ou thérapeutique contre la prééclampsie.

Prévention et traitements de la prééclampsie	Références
Médicamente contre l'hypertention	**Livingston *et al.*, 2003, Scott, 2003, Grill *et al.*, 2009, Altman *et al.*, 2002, Witlin *et al.*, 1998**
Anti-coagulent (ex : aspirine, héparine)	**Duley *et al.*, 2007, Coomarasamy *et al.*, 2001, Askie *et al.*, 2007, Alguel *et al.*, 2006, Sergio *et al.*, 2006, Rey *et al.*, 2009**
Supplément de calcuim	**Atallah *et al.*, 2002, Hofmeyr *et al.*, 2007**
Antioxydant	**Hung *et al.*, 2001, Redman *et al.*, 2009a, Roberts *et al.*, 1999**
Exercice	**Sorensen *et al.*, 2003, Yeo *et al.*, 2008, Yeo *et al.*, 2001**

Tel que discuté précédemment, il a été proposé que le stress oxydatif puisse être un mécanisme causal de la réponse inflammatoire systémique dans la prééclampsie. Des études ont également démontré des niveaux de stress oxydatif plus élevé dans les placentas de grossesses compliquées par la prééclampsie (Burton *et al.*, 2004, Hubel, 1999, Redman *et al.*, 2000, Redman *et al.*, 2009a, Roberts *et al.*, 1999, Vanderlelie *et al.*, 2005). Conséquemment, une thérapie à base d'antioxydant a été proposée. La vitamine C et E sont des chélateurs des radicaux libres et sont retrouvé dans l'alimentation. Ces vitamines permettent de protéger les enzymes, les protéines ainsi que les cellules des dommages causés par le stress oxydatif. La prééclampsie est associée à une diminution de ces antioxydants qui sont nécessaires au maintien des défenses contre le stress oxydatif (Deruelle *et al.*). Les essais cliniques ne supportent pas que l'administration de vitamine C et E puissent prévenir la prééclampsie et propose que ces essais massifs et coûteux cessent, jusqu'à ce que des études plus poussées soient entreprises. Il a été démontré que la combinaison de 1000 mg de vitamine C et 400 IU de vitamine E ne permettent pas de réduire les risques de prééclampsie (Poston *et al.*, 2006). Une

des explications proposées pour l'absence d'effets préventifs ou d'amélioration serait une posologie inadéquate (Reiter *et al.*, 2009). Une extrapolation à partir de modèles animaux suggère que 10-15 g de vitamine C soit requis pour réduire le stress oxydatif chez l'humain (Reiter *et al.*, 2009). De nouvelles approches pour la prévention de la prééclampsie par rapport aux stress oxydatifs incluant des suppléments de mélatonine, de mélatonine conjuguée à la vitamine C, de sélénium, de statines et d'anti-peroxynitrite (Lazebnik *et al.*, 1994, Milczarek *et al.*, 2010, Okatani *et al.*, 2001a, Tamura *et al.*, 2008, Wakatsuki *et al.*, 2001).

HYPOTHÈSE DE RECHERCHE

Le placenta est un organe multifonctionnel qui est indispensable au bon déroulement de la grossesse et de la croissance fœtale. Chez l'espèce humaine, la placentation est accompagnée par une intense activité endocrine du trophoblaste villeux (Malassine *et al.*, 2003). La mélatonine, une hormone au vaste spectre d'activité biologique, possède notamment une puissante action cytoprotectrice de façon dépendante et indépendante de ses récepteurs (Pandi-Perumal *et al.*, 2006). La présence de grande quantité de mélatonine a été démontrée dans le placenta humain, suggérant qu'elle est localement produite (Nakazawa *et al.*, 1999). Chez les femmes enceintes, la concentration plasmatique de mélatonine augmente significativement tout au long de la grossesse pour atteindre un maximum au troisième trimestre, la source de cette augmentation demeure inconnue (Kivela, 1991, Nakamura *et al.*, 2001). Mes travaux de maîtrise ont démontré la présence de récepteurs de la mélatonine dans le tissu placentaire humain normal à terme, sans toutefois préciser le type de cellules les exprimant (Lanoix *et al.*, 2006). Enfin, dans la prééclampsie, une complication de la grossesse dont la pathogénèse implique une perturbation de l'homéostasie du trophoblaste villeux résultant d'une augmentation de ses niveaux d'apoptose (Heazell *et al.*, 2008, Ishihara *et al.*, 2002), les taux sanguins maternels de mélatonine sont significativement diminués en comparaison à la grossesse normotensive (Nakamura *et al.*, 2001).

Ce projet de recherche repose donc sur **l'hypothèse** que la mélatonine joue un rôle protecteur dans le maintien de la survie du trophoblaste villeux, essentiel au bon déroulement de la grossesse, et qu'une altération de la signalisation ou de la production placentaire de mélatonine est impliquée dans la pathogénèse de la grossesse compliquée par la prééclampsie.

Les trois **objectifs** spécifiques pour vérifier cette hypothèse sont : **1**) déterminer la production de mélatonine et l'expression de ses récepteurs dans le placenta humain à terme; **2**) *in vitro,* déterminer la capacité de la mélatonine à prévenir l'apoptose mitochondriale du syncytiotrophoblaste induite par de l'hypoxie/réoxygénation; **3**) comparer la production de mélatonine et l'expression de ses récepteurs dans des placentas de grossesses normotensives et compliquées par la prééclampsie.

Effet protecteur de la mélatonine sur l'homéostasie du trophoblaste villeux : implications dans la grossesse normale et compliquée par la prééclampsie

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Résultats

CHAPITRE 1: HUMAN PLACENTAL TROPHOBLAST SYNTHESIZE MELATONIN AND EXPRESS ITS RECEPTORS

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HUMAN PLACENTAL TROPHOBLASTS SYNTHESIZE MELATONIN AND EXPRESS ITS RECEPTORS

Résumé de l'article en français

Bien que le rôle de la mélatonine sur le développement fœtal ait été l'objet d'un certain nombre d'études, peu de choses sont connues sur les fonctions placentaires de la mélatonine. Nous avons précédemment démontré que les récepteurs de la mélatonine sont exprimés et fonctionnels dans les lignées cellulaires JEG-3 et BeWo, des modèles *in vitro* de trophoblaste humain. La synthèse de mélatonine dans le placenta a été proposée, mais la capacité du placenta humain à synthétiser de la mélatonine *de novo* n'a jamais été étudiée. Le but de cette étude était d'étudier l'expression et l'activité des enzymes synthétisant la mélatonine (essais radiométriques), et de caractériser l'expression des récepteurs mélatoninergiques dans le trophoblaste villeux humain à terme. Les résultats montrent que les enzymes de synthèse de la mélatonine, l'aralkylamine N-acétyltransférase et l'hydroxyindole O-méthyltransférase, sont exprimées et actives dans les cytotrophoblastes villeux, le syncytiotrophoblaste ainsi que dans les lignées cellulaires de choriocarcinomes placentaires JEG-3 et BeWo. De plus, des analyses d'immunohistochimie démontrent la présence des protéines des récepteurs de la mélatonine MT1, MT2 et du récepteur nucléaire orphelin relié aux rétinoïdes RORα dans les cytotrophoblastes villeux et le syncytiotrophoblaste ainsi que dans les cellules endothéliales entourant les capillaires fœtaux et le mésenchyme. Les analyses de RT-PCR et d'immunobuvardage de type Western dans les cultures primaires de trophoblaste villeux humain à terme confirment l'expression des trois récepteurs de la mélatonine dans les cytotrophoblastes villeux et le syncytiotrophoblaste. Cette étude démontre pour la première fois une synthèse locale de mélatonine et une expression de ses récepteurs dans le trophoblaste humain et indique fortement un rôle paracrine, autocrine et/ou intracrine de cette indolamine dans les fonctions et le développement placentaire, telle qu'une protection contre le stress oxydatif.

Contribution de l'étudiant

L'étudiant a réalisé toutes les expériences et l'analyse des résultats présentés dans cet article, contribué à la rédaction de l'article, participé au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

CHAPITRE 2: MELATONIN: THE WATCHDOG OF VILLOUS TROPHOBLAST HOMEOSTASIS AGAINST HYPOXIA/REOXYGENATION-INDUCED OXIDATIVE STRESS AND APOPTOSIS

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MELATONIN: THE WATCHDOG OF VILLOUS TROPHOBLAST HOMEOSTASIS AGAINST HYPOXIA/REOXYGENATION-INDUCED OXIDATIVE STRESS AND APOPTOSIS

Résumé de l'article en français

Le placenta humain produit de la mélatonine et exprime ses récepteurs. Nous proposons que la mélatonine, un antioxydant, protège le syncytiotrophoblaste des dommages cellulaires induits par l'hypoxie/réoxygénation (H/R). Des cytotrophoblastes villeux ont été mis 72 h en culture sous normoxie (8% O_2) en présence ou absence de 1 mM de mélatonine pour induire leur différenciation en syncytiotrophoblaste. Les trophoblastes ont ensuite été cultivés pour une période additionnelle de 22 h sous normoxie ou soumise à une hypoxie (0,5% O_2) pour 4 h suivi d'une réoxygénation (8% O_2) pendant 18 h en présence ou absence de mélatonine. L'H/R induit un stress oxydatif, qui induit l'activation de la voie de Bax/Bcl-2 ainsi que la fragmentation de l'ADN subséquente. Le traitement du syncytiotrophoblaste avec 1 mM de mélatonine renverse significativement tous les effets négatifs induits par l'H/R au niveau de la normoxie. Cette étude montre que la mélatonine protège le syncytiotrophoblaste contre le stress oxydatif et l'apoptose induits par l'H/R. Ces résultats révèlent la potentielle utilisation préventive et thérapeutique de la mélatonine dans les complications de la grossesse caractérisée par une altération de la survie du syncytiotrophoblaste, telle que la prééclampsie.

Contribution de l'étudiant

L'étudiant a réalisé les expériences et analysé les résultats portant sur les figures 5 à 9 présentées dans cet article. Il a participé à la rédaction et révision de l'article et au choix du journal de publication.

Melatonin: the watchdog of villous trophoblast homeostasis against hypoxia/reoxygenation-induced oxidative stress and apoptosis

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Abbreviated title: Melatonin protects the human placenta

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ABSTRACT

Human placenta produces melatonin and expresses its receptors. We propose that melatonin, an antioxidant, protects the human placenta against hypoxia/reoxygenation (H/R)-induced damage. Primary villous cytotrophoblasts were cultured under normoxia (8% O2) with or without 1 mM melatonin for 72 h to induce differentiation into the syncytiotrophoblast. The cells were then cultured for an additional 22 h under normoxia or subjected to hypoxia (0.5% O2) for 4 h followed by 18 h reoxygenation (8% O2) with or without melatonin. H/R induced oxidative stress, which activated the Bax/Bcl-2 mitochondrial apoptosis pathway and the downstream fragmentation of DNA. Syncytiotrophoblast treatment with melatonin reversed all the negative effects induced by H/R to normoxic levels. This study shows that melatonin protects the syncytiotrophoblast against H/R-induced oxidative stress and apoptosis and suggests a potential preventive and therapeutic use of this indolamine in pregnancy complications characterized by syncytiotrophoblast survival alteration.

KEYWORDS: syncytiotrophoblast /reactive oxygen species / antioxidant / mitochondrial apoptosis

ABBREVIATION LIST

Catalase (CAT), Glutathione peroxidase (GPx), Hypoxanthine phosphoribosyltransferase 1 (HPRT1), Hypoxia inducible factor 1 (HIF-1), Hypoxia/reoxygenation (H/R), Nuclear factor-κB (NF-κB), Peptidylprolyl isomerase A (PPIA), Poly(ADP-ribose) polymerase (PARP), Quantitative polymerase chain reaction (real-time PCR; qPCR), Reactive oxygen species (ROS), Reverse-transcription (RT), Rho-associated coiled-coil protein kinase 1 (ROCK-1), Soluble fms-like tyrosine kinase 1 (sFlt-1), Superoxide dismutase 1 (SOD1 or Cu, ZN-SOD), Superoxide dismutase 2 (SOD2, Mn-SOD), Syncytiotrophoblast microparticles (STBM), Tumor necrosis factor α (TNF-α), Xanthine dehydrogenase (XDH), Xanthine oxidase (XO).

1. INTRODUCTION

The maintenance of villous trophoblast homeostasis is essential to fetal and pregnancy well-being. The villous trophoblast is formed by proliferative stem cytotrophoblasts that exit the cell cycle and terminally differentiated into syncytiotrophoblast. The syncytiotrophoblast (multinuleated) is formed by fusion of the underlying mononuclear villous cytotrophoblast which retain the ability to proliferate and fuse with the overlying syncytium allowing contiuous regeneration of the syncytiotrophoblast throughout the pregnancy (Black, Kadyrov, Kaufmann et al., 2004,Vaillancourt, Lanoix, Le Bellego et al., 2009). Therefore, the syncytiotrophoblast must undergo apoptosis to be replaced and maintain its homeostasis (Gauster, Moser, Orendi et al., 2009,Huppertz, Frank, Reister et al., 1999,Lanoix, Lacasse, Reiter et al., 2012). Increased apoptosis in the syncytiotrophoblast disrupts its homeostasis and stimulates the release of syncytial factors in maternal circulation, including tumor necrosis factor α (TNF-α), soluble fms-like tyrosine kinase 1 (sFlt-1) and syncytiotrophoblast microparticles (STBM), which are involved in the pathogenesis of pregnancy complications, such as preeclampsia and intra-uterine growth restriction (Heazell, Sharp, Baker et al., 2011,Ishihara, Matsuo, Murakoshi et al., 2002,Rampersad and Nelson, 2007,Scifres and Nelson, 2009,Tomas, Prusac, Roje et al., 2011).

Placental oxidative stress is a potent inducer of syncytiotrophoblast apoptosis through the mitochondrial pathway (Cindrova-Davies, Spasic-Boskovic, Jauniaux et al., 2007,Heazell, Lacey, Jones et al., 2008,Tuuli, Longtine and Nelson, 2011). Oxidative stress occurs when the production of reactive oxygen species (ROS) overwhelms the intrinsic antioxidant defense system leading to molecular damage (Burton and Jauniaux, 2011). Endogenous homeostatic ROS levels are tightly regulated by the major antioxidant enzymes, including superoxide dismutase 1 (SOD1 or Cu, Zn-SOD), superoxide dismutase 2 (SOD2, Mn-SOD), glutathione peroxidase (GPx) and catalase (CAT), all of which are present in human placenta. At homeostatic levels, ROS act as second messengers to modulate cellular functions, such as proliferation and differentiation and low levels of ROS are necessary to the formation for the syncytiotrophoblast (Robins, Heizer, Hardiman et al., 2007). In addition, over-expression of SOD1 in villous cytotrophoblasts decreases differentiation and syncytial fusion (Frendo, Therond, Bird et al.,

2001). However, at higher ROS levels, the balance between pro-oxidant and antioxidant activities are lost and deleterious outcomes, such as increased syncytiotrophoblast apoptosis, occurs (Cindrova-Davies et al., 2007).

Hypoxia/reoxygenation (H/R) is a potent stimulus for apoptosis of the syncytiotrophoblast via the mitochondrial pathway through altered equilibrium between pro-oxidant and antioxidant defenses (Cindrova-Davies, Yung, Johns et al., 2007,Hung, Skepper, Charnock-Jones et al., 2002,Rampersad and Nelson, 2007,Scifres and Nelson, 2009). Consequently, H/R is a model of choice to study alterations of syncytiotrophoblast homeostasis. In H/R, pro-oxidants are increased through the irreversible conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO). During reoxygenation, XO generates large amounts of ROS which are harmful to cells (Nishino, Nakanishi, Okamoto et al., 1997). In placenta, XO is one of the main generators of ROS (Many, Westerhausen-Larson, Kanbour-Shakir et al., 1996). Under H/R, altered pro-oxidant-antioxidant balance activates redox-sensitive transcription factors such as nuclear factor-kappa B (NF-κB) (Haddad, 2002). NF-κB directly targets p53, a master regulator of cell fate, which triggers apoptosis through the mitochondrial pathway. ROS-induced DNA damage also triggers p53 activation. Key components of the mitochondrial-mediated apoptosis pathway involved an increase of the Bax/Bcl-2 ratio as well as activation of the caspase 9 and caspase 3 (Zamzami and Kroemer, 2001). Cellular manifestations of apoptosis are the formation of membrane blebs and the inhibition of DNA repair mediated by the caspase 3-dependent cleavage of Rho-associated coiled-coil protein kinase 1 (ROCK-1) and poly(ADP-ribose) polymerase (PARP), respectively (Coleman, Sahai, Yeo et al., 2001,Lazebnik, Kaufmann, Desnoyers et al., 1994). Cell death through apoptosis is also characterized by ~~the~~ caspase 3-mediated DNA fragmentation and degradation (Lakhani, Masud, Kuida et al., 2006).

Melatonin reduces H/R-induced damage in many organs, including during myocardial infarction (Tan, Manchester, Reiter et al., 1998), stroke (Koh, 2012) and liver injury due of its powerful antioxidant properties (Kang, Koh and Lee, 2011, Okatani, Wakatsuki, Shinohara et al., 2001, Watanabe, Hamada, Wakatsuki et al., 2012). Melatonin and its metabolites are excellent ROS scavengers (Galano, Tan and

Reiter, 2011). Moreover, melatonin increases the expression and activity of the antioxidant enzymes by mechanisms likely dependent on its MT1 and MT2 receptors (Richter, Hansell, Raut et al., 2009, Rodriguez, Mayo, Sainz et al., 2004). Due to its potent antioxidant properties, melatonin decreases mitochondrial-dependent apoptosis through inhibition of oxidative stress-activated pathways (Das, Belagodu, Reiter et al., 2008, Das, McDowell, Pava et al., 2010, Li, Nickkholgh, Yi et al., 2009), thereby maintaining tissue homeostasis.

We have demonstrated high melatonin production and expression of its receptors in villous trophoblast cells from normal term placentas (Lanoix, Beghdadi, Lafond et al., 2008), suggesting an autocrine and paracrine role. In addition, we have recently shown that melatonin promotes syncytiotrophoblast survival (Lanoix et al., 2012). However, the effect of melatonin on H/R-induced villous trophoblast homeostasis alteration, namely on the increased oxidative stress and mitochondrial apoptosis, has never been studied. The goal of the current study was to determine the effect of melatonin on villous trophoblast subjected to normoxia and H/R on: i) ROS generation, ii) expression and activity of antioxidant enzymes, iii) activation of ROS-induced cell signaling pathway, and, iv) induction of mitochondrial apoptosis.

2. MATERIAL AND METHODS

2.1 Isolation, purification and treatment of term villous trophoblasts

This study was approved by the ethical committee of CHUM-St-Luc Hospital (Montreal, QC, Canada). Villous cytotrophoblasts from term placentas of uncomplicated pregnancies were isolated and purified as previously described by our group (Lanoix et al., 2008,Lanoix and Vaillancourt, 2010,Le Bellego, Vaillancourt and Lafond, 2009), using the trypsin-DNase/Percoll method (Kliman, Nestler, Sermasi et al., 1986). Mononuclear villous cytotrophoblasts were purified by immunomagnetic labelling using autoMACS™ (Myltenyi Biotec, Auburn, CA, USA) and anti-HLA-ABC antibody as described previously (Lanoix et al., 2008).

Villous cytotrophoblasts (n = 3 different placentas) were cultured for 96 h under three different oxygen conditions in Modular Incubator Chambers (Billups-Rothenberg, Del Mar, CA, USA). Cells were seeded at a density of 5.75 x 10^5 cells/cm^2 and cultured for 6 h in normoxia (8% O_2, 5% CO_2, 87% N_2) to allow adherence and then subjected to: a) normoxia, control condition, b) H/R or c) H/R with 1 mM melatonin treatment as described in **Figure 1**. As they do *in vivo*, *in vitro* mononuclear villous cytotrophoblast cells fused and differentiate into a syncytium, the syncytiotrophoblast. To verify if melatonin treatment affect the rate of syncytialization of primary cytotrophoblast cells prior to the induction of H/R, hCG production in cell culture supernatant was monitored daily, from 0 to 72 h, using an hCG enzyme-linked immunosorbent (ELISA) from DRG Diagnostics as described previously (Lanoix et al., 2008,Le Bellego et al., 2009) (**Supplemental data 1**). Melatonin was dissolved in DMSO at a final concentration of 0.1%. Normoxia (0.1% DMSO as vehicle) was established at 8% O_2, a concentration that is maintained in normal pregnancy from 12 weeks gestation to term (Schneider, 2011). Hypoxic oxygen concentration of 0.5% and 16 h of reoxygenation were used as established by Cindrova-Davies *et al.* to promote mitochondrial apoptosis (Cindrova-Davies et al., 2007). Chambers were flushed for 4 min at 25 L/min with a mixture of O_2, CO_2 and N_2 to achieve the desired final oxygen concentration (8% or 0.5%) and a final CO_2 concentration of 5%. Dissolved O_2 at the cell-medium interface was confirmed using a microoxygen electrode (MI-730; Microelectrodes Inc., Londonderry, NH, USA). All media were pre-equilibrated with the appropriate gas mixture before addition to the culture plate and refreshed every 24 h.

2.2 mRNA expression analysis

The reverse-transcription (RT) quantitative polymerase chain reaction (real-time PCR; qPCR) analysis was performed as described previously (Lanoix, Lacasse, St-Pierre et al., 2012,Lanoix, Lacasse, St-Pierre et al., 2012). Briefly, total RNA was extracted from primary villous trophoblast after 96h of culture using Aurum Total RNA mini kit according to manufacturer's instructions (Bio-Rad, Mississauga, Canada). RNA was quantified by spectrophotometric measurement (Spectramax, Molecular Devices). The Experion™ Automated Electrophoresis Station (Bio-Rad) was used to determine RNA integrity.

Complimentary DNA (cDNA) was obtained from 2 µg RNA with the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad) and stored at -20°C until further analysis. Specific primer for XO, SOD1, SOD2, GPx, CAT, HPRT1 and PPIA were designed using Oligo 6 software (Molecular Biology Insights, Cascade, USA) and their specificity was determined with Primer-Blast program (http://www.ncbi.nlm.nih.gov/tools/primerblast/). Validated primer sequences are shown in **Table 1**. HPRT1 and PPIA were selected as reference genes according to the method described in Lanoix *et al.*(Lanoix et al., 2012,Lanoix et al., 2012).

qPCR reactions were conducted using the CFX-96 Real-Time PCR Detection System (Bio-Rad). Amplification was performed with SsoFast EvaGreen Supermix (Bio-Rad) from 0.5 µl of cDNA as described previously (Lanoix et al., 2012).

2.3 Proteins expression analysis

Briefly, cells were collected with ice-cold modified radioimmunoprecipitation (RIPA) buffer (50 mmol/l Tris-HCL pH 7.4, 1% NP-40, 0,25% nadeoxycholate, 150 mmol/l NaCL and 1 mmol/l EDTA) containing protease and phosphatase inhibitors (Sigma-Aldrich, St-Louis, MO, USA) and then sonicated as described (Lanoix, Ouellette and Vaillancourt, 2006,Lanoix, Beghdadi, Lafond et al., 2008). Samples were homogenised using a QIAshredder spin column homogenizer (Qiagen, Toronto, Canada). The homogenate was then centrifuged at 13 000 g for 10 min at 4 °C to remove insoluble material. Protein concentration of each sample was determined by spectrophotometric quantification (SpectraMax M5 with SoftMax Pro v5 software, Molecular Device, Sunnyvale, CA, USA) using the bicinchoninic acid (BCA) protein assay reagent according to manufacturer's instruction (Pierce Biotechnology, Rockford, IL, USA).

Proteins (50 µg per lane) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 12% separating gels), and were subsequently transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Mississauga, ON, Canada) as described previously (Lanoix et al., 2008). Specific primary antibodies are described in **Table 2**. Band intensities were analyzed and quantified with AlphaEaseFC software (Alpha Innotech). After the antibody procedures, membranes were stained with

amido black to normalize target protein expression as previously described (Lanoix, St-Pierre, Lacasse et al., 2012). In some cases, blots were stripped with Re-Blot Plus Mild Antibody Stripping Solution, rinsed twice with TBST and reprobed with another primary antibody.

2.4 Relative ROS levels analysis

Relative ROS levels were analyzed with the Reactive Oxygen Species Detection Reagents (Invitrogen) that detect hydrogen peroxide, peroxyl radical, peroxynitrite anion and superoxide anion, all of which are scavenged by melatonin. After 96 h of culture, primary villous trophoblasts were rinsed and incubated with 10 μM of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) pre-equilibrate at 8% oxygen, for 45 min in the Modular Incubator Chambers. After rinsing, relative ROS levels were determined by fluorimetry according to manufacturer's instruction and normalized against protein content of cell culture well.

2.5 SOD, GPx and XO activity assays

Samples were prepared from primary villous trophoblast after 96 h of culture. Enzyme activity assays were performed using SOD Activity Assay Kit # K335-100, GPx Activity Assay Kit #K762-100 and XO Assay Kit #K710-100 according to manufacturer's instruction (BioVision, Mountain View, USA). Cells were homogenized in cold Assay Buffer and centrifuged to remove debris. Enzyme activity was determined spectrophotometrically in supernatants using specific standard curve.

2.6 DNA fragmentation analysis

Internucleosomal DNA fragmentation was evaluated to detect villous trophoblast apoptosis by standard agarose gel electrophoresis as previously described (Dupere-Minier, Hamelin, Desharnais et al., 2004). Briefly, primary villous trophoblasts after 96 h of culture were washed twice with ice-cold PBS, resuspended in 20 μl of 10 mM EDTA, 50 mM Tris-HCl (Ph 8.0) containing 0.5 % M/W sarkosyl, 0.5

mg/ml proteinase K and incubated at 50°C for 1.5 h. One µl of 30 mg/ml RNase A was added to samples and further incubated for 1 hour at 50°C. Samples were then heat at 70°C for 10 min, loaded on a 2% agarose gel and electrophoresed for 1 h at 100 V. DNA was stained with ethidium bromide and visualized by UV transillumination using a FluorChem FC2 (Alpha Innotech).

2.7 Statistical analysis

All data are represented as mean ± S.D. from three different villous trophoblast cells isolations. Statistical significance was determined by one-way ANOVA followed by Tuckey-Kramer *post hoc* comparison test using Prism 5.0 (GraphPad, San Diego, CA, USA). Data were considered statistically significant at a value of $P \leq 0.05$.

3 RESULTS

3.1 Melatonin prevents the increased oxidative stress induced by H/R

Carboxy-H2DCFDA fluorescence showed that H/R significantly increased ROS levels compared to normoxia ($P = 0.0283$) and this effect was reversed by melatonin treatment ($P = 0.0152$) (**Fig. 2A**). Considering that melatonin is a powerful antioxidant (Galano et al., 2011) and that basal ROS levels are required to allow syncytiotrophoblast differentiation (Robins et al., 2007), ROS levels were determined between normoxia and normoxia combined with melatonin treatment. **Supplementary data 2A** shows that melatonin maintains basal ROS level required for its differentiation under normoxia. The expression and activity of XO, the main generator of ROS in the placenta, was determined by RT-qPCR and semi-quantitative western blot as well as by colorimetric assay respectively. **Figure 2B-D** shows that H/R significantly increased XO mRNA ($P = 0.0212$) and protein ($P = 0.0346$) expression as well as its activity ($P = 0.0134$) compared to normoxia. Melatonin significantly reverses the effect of H/R on XO mRNA ($P = 0.0443$) and protein ($P = 0.0345$) expression as well as its activity ($P = 0.0235$). This indicates that melatonin significantly reverses the increased pro-oxidant level induced by H/R. Therefore, to determine the pro-oxidant-antioxidant balance, the antioxidant defence enzymes were analyzed.

3.2 Melatonin prevents the alteration of antioxidant enzymes induced by H/R

SOD1 and SOD2 mRNA ($P = 0.0088$ and $P = 0.0451$, respectively) and protein ($P = 0.0013$ and $P = 0.0042$, respectively) expression were significantly decreased under H/R compared to normoxia (**Fig. 3A-D**). **Figure 3A-D** demonstrates that melatonin prevents the H/R-induced decreased SOD1 and SOD2 mRNA ($P = 0.0317$ and $P = 0.0386$, respectively) and protein ($P = 0.0003$ and $P = 0.0151$, respectively) expression. In addition, H/R significantly decreased total SOD activity compared to normoxia ($P = 0.0069$) and this effect was reversed by melatonin ($P = 0.0021$) (**Fig. 3E**). Over-expression of SOD1 in the syncytiotrophoblast under normal condition has been shown to decrease its differentiation (Frendo et al., 2001). Therefore, the effect of melatonin on the expression of SOD1 and SOD2 in normoxia was characterized to ensure that there are no deleterious effects of melatonin. **Supplementary data 2B and 2C** show that in the syncytiotrophoblast under normoxia, melatonin has no effect on the expression of SOD1 and SOD2 protein.

Figure 3F to 3H demonstrates a significant reduction of GPx mRNA ($P = 0.0025$) and protein ($P = 0.0015$) expression as well as its activity ($P = 0.0105$) under H/R compared to normoxia. Melatonin treatment significantly reverses these effects of H/R on GPx activity ($P = 0.0483$) as well as its mRNA ($P = 0.0250$) and protein ($P = 0.0033$) expression. **Figure 3** demonstrates alterations in endogenous antioxidant defence enzymes under H/R, resulting in increased intracellular ROS levels, and the capacity of melatonin to significantly prevent these deleterious effects. Given that ROS induced mitochondrial apoptosis through activation of redox-sensitive transcriptions factors, including NF-κB and p53, their expression was investigated under H/R combined or not with melatonin treatment.

3.3 Melatonin prevents the expression of the active form of redox-sensitive signaling pathways induced by H/R

H/R induces significant expression of the stable form of HIF-1 ($P = 0.0005$) in the syncytiotrophoblast compared to normoxia which is significantly inhibited ($P = 0.0023$) by melatonin

(**Fig. 4A**). **Figure 4B and 4C** demonstrate an increased expression of the active form of NF-κB p65 protein ($P = 0.0004$) and the subsequent induction of p53 ($P = 0.0063$) under H/R compared to normoxia. These effects were significantly reduced by melatonin (NF-κB; $P = 0.0002$ and p53; $P = 0.0059$). These results demonstrate the ability of melatonin to prevent the activation of signaling pathways triggering mitochondrial apoptosis induced by H/R in primary syncytiotrophoblast. Consequently, the expression of key components of the mitochondrial apoptosis cascade and of associated syncytial factors was studied.

3.4 Melatonin prevents the stimulation of mitochondrial apoptosis and of associated syncytial factors induced by H/R

Primary syncytiotrophoblast under H/R displays a significantly increased Bax/Bcl-2 ratio, an indicator of mitochondrial apoptosis activation, compared to normoxia ($P = 0.0074$) while melatonin treatment prevents it ($P = 0.0086$, **Fig. 5A**). **Figure 5B and 5C** demonstrate that H/R induces the cleavage of caspase-9 ($P = 0.0008$) and caspase-3 ($P = 0.0024$) compared to normoxia in the syncytiotrophoblast while melatonin significantly reverses their activation (caspase-9, $P = 0.0087$; caspase-3, $P = 0.0073$). In villous trophoblast, H/R mediates significant caspase 3-dependent cleavage of ROCK-1 ($P = 0.0053$) and PARP ($P = 0.0046$) compared to normoxia (**Fig. 5D-E**). H/R-induced cleavage of ROCK-1 ($P = 0.0064$) and PARP ($P = 0.0395$) are significantly inhibited by melatonin (**Fig. 5D-E**). **Figure 5F** demonstrates increased DNA fragmentation under H/R compared to normoxia whereas melatonin treatment decreased DNA fragmentation to a level similar to normoxia. Taken together, **figure 5** demonstrates that in the syncytiotrophoblast under H/R, apoptosis by the mitochondrial pathway is significantly prevented by 1 mM melatonin. The syncytiotrophoblast under normal conditions requires basal level of apoptosis to allow its regeneration and thus maintain its homeostasis (Huppertz et al., 1999). Thus, DNA fragmentation was compared between normoxia and normoxia combined with melatonin treatment. Melatonin has no effect on the level of DNA fragmentation in normoxia, and therefore ~~of~~ on apoptosis, required to maintain syncytiotrophoblast homeostasis (**Supplementary data 2D**).

To evaluate the potential of melatonin to decrease the production of factors from the syncytiotrophoblast consequent to its apoptosis, we investigated the expression of TNF-α. **Supplementary data 3** demonstrated that H/R significantly increased the expression of TNF-α (P = 0.0003) compared to normoxia and this effect was reversed by melatonin treatment TNF-α (P = 0.0002). These observations are in accordance with the inhibition of H/R-induced mitochondrial apoptosis by melatonin.

4. DISCUSSION

This study demonstrates a protective effect of melatonin against mitochondrial-mediated apoptosis of the syncytiotrophoblast induced by H/R through maintenance of the equilibrium between pro-oxidants and antioxidants. These results suggest that melatonin plays a crucial role in placental functions and pregnancy well-being by maintaining syncytiotrophoblast homeostasis.

Normal pregnancy is itself a state of oxidative stress (Wisdom, Wilson, McKillop et al., 1991). In addition, oxidative stress and apoptosis of the syncytiotrophoblast are normal physiological processes in the human placenta (Huppertz et al., 1999,Jauniaux, Watson, Hempstock et al., 2000). However, deleterious effects, such as activation of redox-sensitive transcription factors triggering the mitochondrial-dependent apoptosis pathway, occur when the endogenous antioxidants are overwhelmed by increasing ROS levels (Cindrova-Davies et al., 2007). Such exacerbated placental oxidative stress and apoptosis levels are associated with the pathogenesis of intra-uterine growth restriction and preeclampsia (Ishihara et al., 2002,Tomas et al., 2011). In this study, primary villous trophoblasts challenged with H/R *in vitro* showed a marked increase in oxidative stress, activation of redox-sensitive transcription factors and the downstream rise in mitochondrial apoptosis (**Fig. 6**). In accord with our results, it has been demonstrated that the generation of oxidative stress under H/R arises through elevated ROS production and decreased antioxidant enzyme defences in the syncytiotrophoblast (Cindrova-Davies et al., 2007,Hung, Skepper and Burton, 2001). As we observed, others have shown abnormally high ROS levels under H/R are activators of redox-sensitive transcription factors, such as NF-κB and HIF-1 (Cindrova-Davies et al., 2007,Jung,

Isaacs, Lee et al., 2003), which triggers the mitochondrial apoptotic cascade in the syncytiotrophoblast and other tissues (Cindrova-Davies et al., 2007,Hung and Burton, 2006). H/R has been selected over hypoxia alone as an inducer of syncytiotrophoblast apoptosis because it initiates villous trophoblast alterations of pro-oxidant-antioxidant balance occurring in several pregnancy complications, such as preeclampsia and intra-uterine growth restriction (Hung et al., 2001,Hung et al., 2002,Hung and Burton, 2006).

The present work demonstrates that melatonin significantly limits syncytiotrophoblast oxidative stress on both sides of the equation, by inhibition of ROS generation and by restoration of antioxidant enzyme defences (**Fig. 6**). As observed in this study, melatonin is a powerful antioxidant acting through inhibition of pro-oxidant levels and promotion of antioxidant defences (Rodriguez et al., 2004). Melatonin also significantly reduces H/R-induced mitochondrial-mediated apoptosis of the syncytiotrophoblast. In agreement with these results, melatonin has been reported to inhibit the activation of redox-sensitive transcription factors, including NF-κB and HIF-1, and the associated rise in mitochondrial apoptosis (Das et al., 2008, Das et al., 2010). The current results show for the first time, that melatonin reverses the activation of redox-sensitive transcription factors as well as the induction of mitochondrial-dependent apoptosis arising from abnormally high oxidative stress. Studies have proposed the utility of melatonin to combat trophoblast oxidative stress damage during pregnancy (Aversa, Pellegrino, Barberi et al., 2012); however, we provided the first data supporting this assertion. Additional studies on the effect of melatonin against oxidative stress and apoptosis in syncytiotrophoblast under H/R are required to further characterize the cytoprotective role of melatonin in the placenta, for example, on lipid peroxydation and the other pathways of mitochondrial apoptosis, such as apoptosis inducing factor (AIF), Endo G and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein binding protein with a low pI (Smac/DIABLO) (Lanoix et al., 2012).

We have recently demonstrated reduced melatonin production and expression of its receptors in preeclamptic placentas compared to normotensive pregnancies (Lanoix, Guerin and Vaillancourt, 2012), suggesting a role of melatonin in the pathogenesis of preeclampsia and thus its potential clinical utility in

women with decreased plasma level of melatonin. Accordingly, melatonin significantly reduces many placental alterations found in preeclampsia analyzed in this study, including H/R-induced ROS generation (Wang and Walsh, 2001), increased expression and activity of XO (Many, Hubel, Fisher et al., 2000), decreased expression and activity of SOD and GPx (Vanderlelie, Venardos, Clifton et al., 2005,Wang and Walsh, 2001), induction of ROS-activated pathways (Aban, Cinel, Arslan et al., 2004), activation of the Bax/Bcl-2 mitochondrial apoptosis pathway leading to caspase 9 and 3 activation as well as DNA fragmentation (Leung, Smith, To et al., 2001). In addition, we show that melatonin prevents syncytial factor expression induced by H/R, such as TNF-α and HIF-1, which are responsible for the maternal syndrome of preeclampsia. Antioxidants, such as vitamin C and E, have been used to limit H/R-induced damage in preeclampsia. However, despite their reported *in vitro* efficiency against H/R-induced villous trophoblast damage (Cindrova-Davies et al., 2007), *in vivo*, these vitamins show no benefits in the prevention of preeclampsia (Poston, Briley, Seed et al., 2006,Rumbold, Crowther, Haslam et al., 2006,Tuuli et al., 2011). Proposed explanations for the failure of vitamins to prevent or ameliorate preeclampsia is an inadequate dosage or inaccessibility of the vitamins to the placenta (Reiter, Tan, Manchester et al., 2009). In contrast to vitamins, melatonin is a powerful *in vivo* antioxidant even at low doses (Bubenik, Blask, Brown et al., 1998). A prospective clinical study demonstrates that two hundred-fold higher dosage of vitamin E than melatonin is required to achieve the same protective antioxidant effect (Reiter et al., 2009,Tamura, Takasaki, Miwa et al., 2008). Moreover, melatonin, a lipophilic and hydrophilic indoleamine, enters every subcellular compartment (Venegas, Garcia, Escames et al., 2012) and rapidly crosses every morphophysiological barrier, including the placental barrier (Okatani, Okamoto, Hayashi et al., 1998,Pardridge and Mietus, 1980), in opposition to vitamin E (Bortolotti, Traina, Barzago et al., 1990). Supporting our results, other authors have proposed the use of melatonin to prevent or treat preeclampsia (Lanoix et al., 2012, Milczarek, Hallmann, Sokołowska et al., 2010, Okatani et al., 2001, Wakatsuki, Okatani, Ikenoue et al., 2001). The effect of melatonin on primary villous trophoblast cells isolated from placentas of pregnancies complicated by preeclampsia in comparison to normotensive pregnancies remains unknown. The villous trophoblast is the alleged tissue responsible (Kivela,

1991,Lanoix et al., 2008) for the increased maternal blood melatonin levels occurring during pregnancy (Kivela, 1991, Nakamura, Tamura, Kashida et al., 2001) and those levels are significantly reduced in cases of preeclampsia (Nakamura et al., 2001, Tranquilli, Turi, Giannubilo et al., 2004). Thus, it may be important to characterized *in vitro* melatonin production in syncytiotrophoblast under H/R in comparison to normoxia. In addition to preeclampsia, melatonin could be effectively used in the management of pregnancy complications in case of alterations of villous trophoblast homeostasis, including intra-uterine growth restriction and gestational diabetes mellitus (Heazell et al., 2011). Melatonin has been implicated in the pathogenesis of gestational diabetes mellitus and proposed as a potential treatment for this condition (Figueroa and Agil, 2011).

In conclusion, this work establishes melatonin as a potential watchdog against H/R-induced villous trophoblast homeostasis alterations. This is the first study to ascertain the preventive role of melatonin against oxidative stress damage in the syncytiotrophoblast in placentas from normal pregnancies and to document that melatonin treatment reverses mitochondrial-dependent apoptosis arising from abnormally high oxidative stress. Taken together with our previous work showing a decrease of melatonin in the preeclamptic placenta (Lanoix, Guerin and Vaillancourt, 2012), these results reinforce the potential preventive and therapeutic use of melatonin in pregnancy complications that involve villous trophoblast homeostasis alterations, such as preeclampsia as suggested by us (Lanoix et al., 2012) and others (Milczarek et al., 2010,Okatani et al., 2001,Wakatsuki et al., 2001). While more studies are needed, one thorough investigation conducted by the U.S. Center for Life Sciences and Toxicology has documented the maternal and fetal safety of melatonin during pregnancy when given over a wide range of doses (Jahnke, Marr, Myers et al., 1999).

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Table 1. Genes and primer sequences used for RT-qPCR.

Gene	Accession number	Primer sequence	
		Sense	Antisense
GPx	NM_002083	CAACCAGTTTGGGCATCAGG	AGCATGAAGTTGGGCTCGAAC
HPRT1	NM_000194	GACCAGTCAACAGGGGACATAA	AAGCTTGCGACCTTGACC
PPIA	NM_021130	GTTTGCAGACAAGGTCCCA	ACCCGTATGCTTTAGGATG
SOD1	NM_000454	TGCTGGTTTGCGTCGTAGTCT	CATGCAGGCCTTCAGTCAGTC
SOD2	NM_000636	AGCGGTAGCACCAGCACTAGC	TAGTCGTAGGGCAGGTCGG
XO	NM_000379	TGTTTCATTTCCGCTGATG	ATGCCCAACACAAGTAACCTT

GPx: Glutathione peroxidase; HPRT1: Hypoxanthine phosphoribosyltransferase 1; PPIA: Peptidylprolyl isomerase A; SOD: Superoxide dismutase; XO: Xanthine oxidase.

Table 2: Antibodies used for Western blot analysis.

Antibody	Dilution	Incubation	Source
Primary antibody			
anti- Bax	1:250	O/N at 4°C	Cell Signaling (2774)
anti- Bcl-2	1:250	O/N at 4°C	Cell Signaling (2872)
anti- cleaved caspase 3	1:500	O/N at 4°C	Cell Signaling (9661)
anti- cleaved caspase 9	1:250	O/N at 4°C	Cell Signaling (9501)
anti-GPx	1:500	O/N at 4°C	Abcam (ab22604)
anti-HIF-1	1:2000	O/N at 4°C	Abcam (ab16066)
anti-NF-κB p65	1:250	O/N at 4°C	Millipore (ST1047)
anti-p53	1:250	O/N at 4°C	Biolegend (628202)
anti- cleaved PARP	1:250	O/N at 4°C	Cell Signaling (5625)
anti-ROCK-1	1:100	O/N at 4°C	Cell Signaling (4035)
anti-SOD1	1:2000	O/N at 4°C	Abcam (ab16831)
anti-SOD2	1:2000	O/N at 4°C	Abcam (ab13534)
anti-TNF-α	1:1000	O/N at 4°C	Abcam (ab13179)
anti-XO	1:2000	O/N at 4°C	Abcam (ab6194)
Secondary antibody			
anti-goat-HRP	1:10 000	1 h at RT	Millipore (AP180P)
anti-mouse-HRP	1:10 000	1 h at RT	Millipore (AP192P)
anti-rabbit-HRP	1:10 000	1 h at RT	Millipore (AP182P)

O/N: overnight; RT: room temperature.

FIGURE LEGENDS

Figure 1: Experimental design of villous trophoblast cell culture. Villous cytotrophoblasts were cultured under normoxia (8% O_2) with or without 1 mM melatonin for 72 h to induce their differentiation into the syncytiotrophoblast. The cells were then cultured for an additional 22 h under normoxia or subject to hypoxia (0.5% O_2) for 4 h followed by 18 h reoxygenation (8% O_2) with or without 1 mM melatonin. Oxidative stress and mitochondrial-dependent apoptosis parameters were subsequently analyzed. STB: syncytiotrophoblast; vCTB: villous cytotrophoblasts.

Figure 2: Melatonin prevents hypoxia/reoxygenation (H/R)-induced generation of oxidative stress. (**A**) Relative ROS levels in primary villous trophoblasts were determined using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) fluorometric assay as described in Material and Methods. (**B**) XO mRNA was amplified by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The geometric average of peptidylprolyl isomerase A (PPIA) and hypoxanthine phosphoribosyltransferase (HPRT1) was used to normalize gene expression. (**C**) XO protein expression was determined using semi-quantitative Western blot. Amido black stain was used to normalize protein expression. (**D**) XO activity in cells homogenate was determined by colorimetric assay as described in Material and Methods. RFU: relative fluorescence units; N: normoxia (8% O_2, 5% CO_2, 87% N_2); H/R: hypoxia/reoxygenation (0.5% O_2, 5% CO_2, 94.5% N_2 → 8% O_2, 5% CO_2, 87% N_2); 1 mM melatonin. Results are expressed as mean ± SD; n= 3 different placentas; * $P \leq 0.05$.

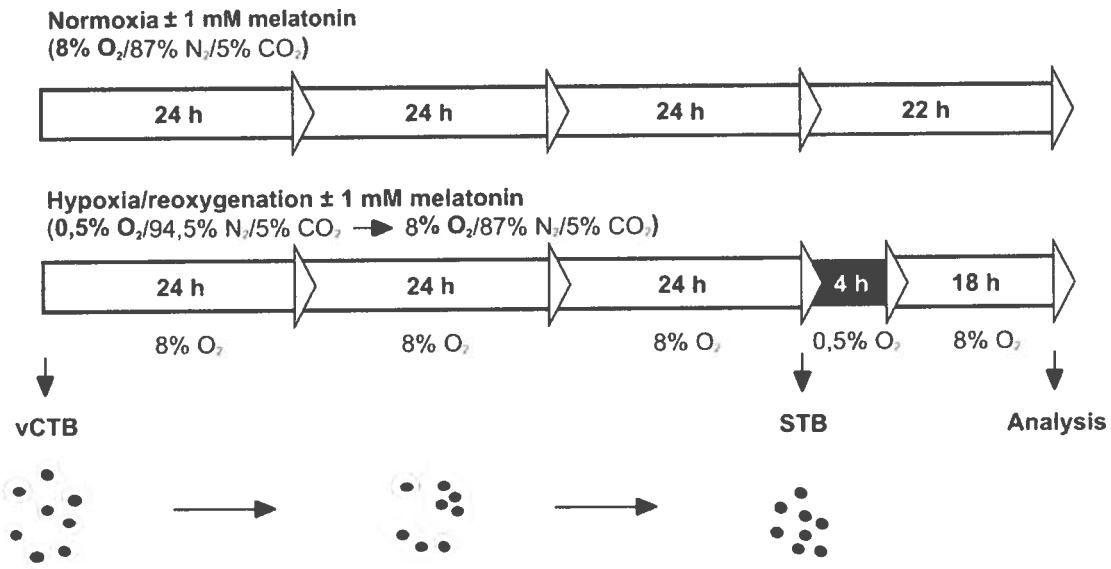
Figure 3: Melatonin prevents hypoxia/reoxygenation (H/R)-induced decreased SOD and GPx expression and activity. (**A**) SOD1, (**B**) SOD2 and (**F**) GPx mRNA was amplified by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The geometric average of PPIA and HPRT1 was used to normalize gene expression. Protein expression of (**C**) SOD1, (**D**) SOD2 and (**G**) GPx was determined using semi-quantitative Western blot. Amido black stain was used to normalize protein

expression. (**E**) Total SOD and (**H**) GPx activity in cells homogenate was determined by colorimetric assay as described in Material and Methods. SOD: superoxide dismutase; GPx: glutathione peroxidase; N: normoxia (8% O_2, 5% CO_2, 87% N_2); H/R: hypoxia/reoxygenation (0.5% O_2, 5% CO_2, 94.5% N_2 → 8% O_2, 5% CO_2, 87% N_2); 1 mM melatonin; PPIA: peptidylprolyl isomerase A, HPRT1: hypoxanthine phosphoribosyltransferase 1. Results are expressed as mean ± SD; n= 3 different placentas; * $P \leq 0.05$; ** $P \leq 0.01$.

Figure 4: Melatonin prevents the expression of the active form of HIF-1, NF-κB p65 and p53 induced by hypoxia-reoxygenation (H/R). Protein expression of, (**A**) HIF-1, (**B**) NF-κB and (**C**) p53 was determined using semi-quantitative Western blot. Amido black stain was used to normalize protein expression. HIF-1: hypoxia inducible factor 1; NF-κB p65: nuclear factor-kappa B p65; N: normoxia (8% O_2, 5% CO_2, 87% N_2); H/R: hypoxia/reoxygenation (0.5% O_2, 5% CO_2, 94.5% N_2 → 8% O_2, 5% CO_2, 87% N_2); 1 mM melatonin. Results are expressed as mean ± SD; n= 3 different placentas; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 5: Melatonin prevents the induction of mitochondrial-mediated apoptosis induced by hypoxia-reoxygenation (H/R). Protein expression of (**A**) Bax relative to Bcl-2 and expression of (**B**) cleaved caspase 9, (**C**) cleaved caspase3, (**D**) ROCK-1 and (**E**) cleaved PARP was determined using semi-quantitative Western blot. Amido black stain was used to normalize protein expression. (**F**) DNA fragmentation was evaluated by agarose gel electrophoresis. Bax: Bcl-2-associate X protein; Bcl-2: B-cell lymphoma 2; ROCK-1: Rho-associated, coiled-coil containing protein kinase 1; PARP: Poly (ADP-ribose) polymerase; N: normoxia (8% O_2, 5% CO_2, 87% N_2); H/R: hypoxia/reoxygenation (0.5% O_2, 5% CO_2, 94.5% N_2 → 8% O_2, 5% CO_2, 87% N_2); 1 mM melatonin. Results are expressed as mean ± SD; n= 3 different placentas; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 6: Proposed anti-apoptotic pathway of melatonin in the syncytiotrophoblast subsequent to hypoxia/reoxygenation. Primary villous cytotrophoblasts were cultured for 72 h under normoxia (8% O_2) to allow the differentiation into syncytiotrophoblast. The cells were then subjected to hypoxia (0.5% O_2) for 4 h followed by 18 h reoxygenation (8% O_2) with or without melatonin as described in Material and Methods. Hypoxia/reoxygenation induces xanthine oxidase (XO) expression and activity, thereby generating reactive oxygen species (ROS). ROS activate redox sensitive transcription factors including hypoxia inducible factor 1 (HIF-1) and nuclear factor kappa B (NF-κB). NF-κB induces p53 which triggers the Bax/Bcl-2 pathway of mitochondrial apoptosis. ROS-induced DNA fragmentation also activated p53. Activation of the Bax/Bcl-2 pathway leads to the activation of caspase 9 and caspase 3. Downstream effects of caspase 3 are the activation of Rho-associated, coiled-coil containing protein kinase 1 (ROCK-1), the cleavage of poly (ADP-ribose) polymerase (PARP) and the DNA fragmentation. Melatonin reverses the induction of mitochondrial apoptosis through reduce oxidative stress. Melatonin passes through all physiological membranes and then scavenges ROS in the cytoplasm, mitochondria and nucleus. Melatonin via its MT1 and MT2 receptors indirectly increase the expression and activity of superoxide dismutase (SOD) 1 and 2 as well as glutathione peroxidase (GPx) antioxidant enzymes. XO expression and activity is indirectly reduced by melatonin and its receptors. Plain line: direct mechanism; dash line: indirect mechanism; arrowhead: stimulation.


Normoxia ± 1 mM melatonin
(8% O2/87% N2/5% CO2)
24 h
24 h
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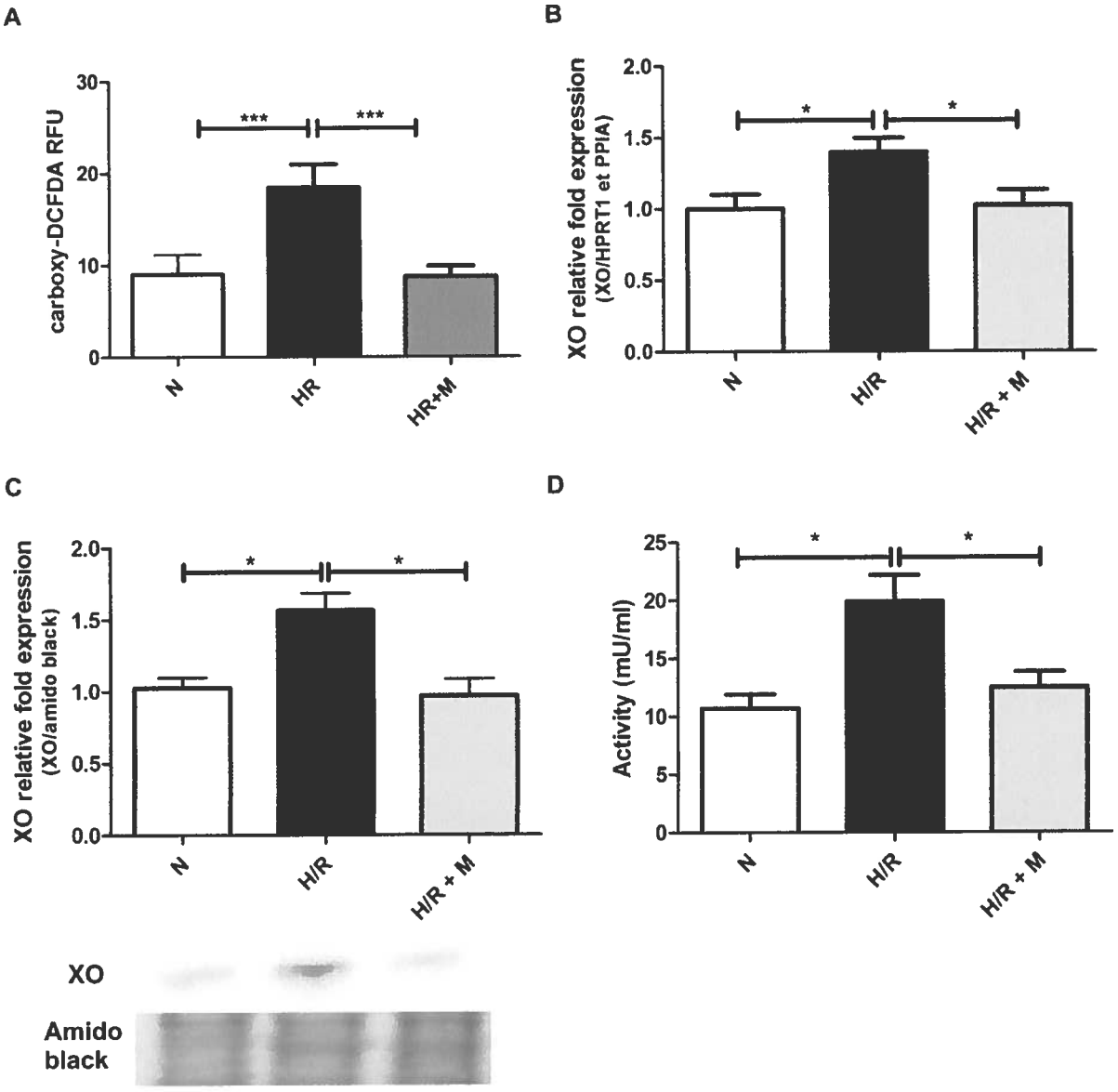
22 h
Hypoxia/reoxygenation ± 1 mM melatonin
(0,5% O2/94,5% N2/5% CO2 → 8% O2/87% N2/5% CO2)
24 h
24 h
24 h
4 h
18 h
8% O2
8% O2
8% O2
0,5% O2
8% O2
vCTB
STB
Analysis

Figure 1.


A
carboxy-DCFDA RFU
30
20
10
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N
HR
HR+M
B
XO relative fold expression
(XO/HPRT1 et PPIA)
2.0
1.5
1.0
0.5
0.0
*
*
N
H/R
H/R + M
C
XO relative fold expression
(XO/amido black)
2.0
1.5
1.0
0.5
0.0
*
*
N
H/R
H/R + M
D
Activity (mU/ml)
25
20
15
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N
H/R
H/R + M
XO
Amido
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Figure 2.

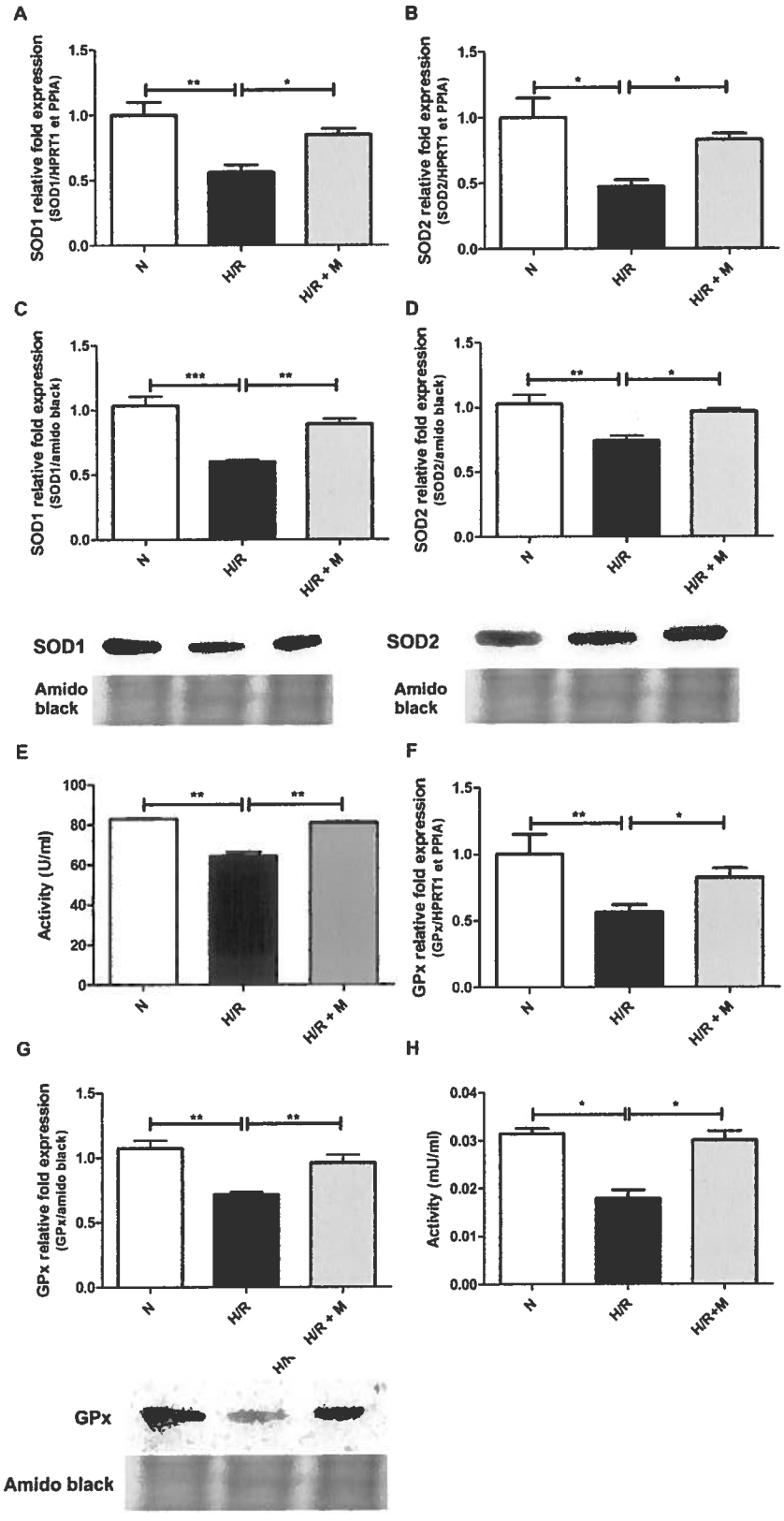

A
SOD1 relative fold expression (SOD1/HPRT1 et PPIA)
B
SOD2 relative fold expression (SOD2/HPRT1 et PPIA)
C
SOD1 relative fold expression (SOD1/amido black)
D
SOD2 relative fold expression (SOD2/amido black)
N
H/R
H/R + M
SOD1
Amido black
SOD2
Amido black
E
Activity (U/ml)
F
GPx relative fold expression (GPx/HPRT1 et PPIA)
G
GPx relative fold expression (GPx/amido black)
H
Activity (mU/ml)
H/R+M
GPx
Amido black

Figure 3.

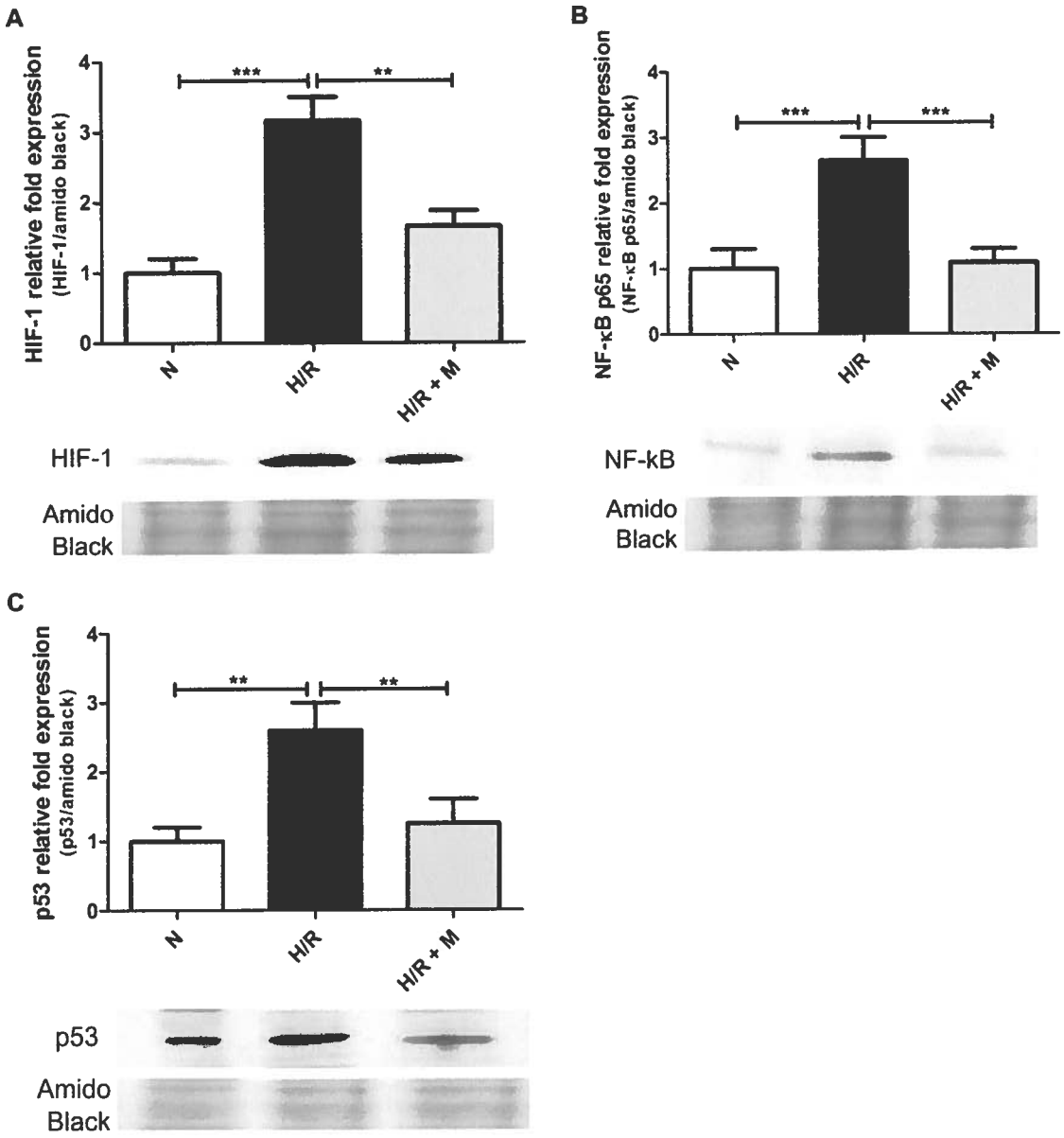

A
HIF-1 relative fold expression
(HIF-1/amido black)
***
**
N
H/R
H/R + M
HIF-1
Amido
Black
B
NF-κB p65 relative fold expression
(NF-κB p65/amido black)
***
***
N
H/R
H/R + M
NF-kB
Amido
Black
C
p53 relative fold expression
(p53/amido black)
**
**
N
H/R
H/R + M
p53
Amido
Black

Figure 4.

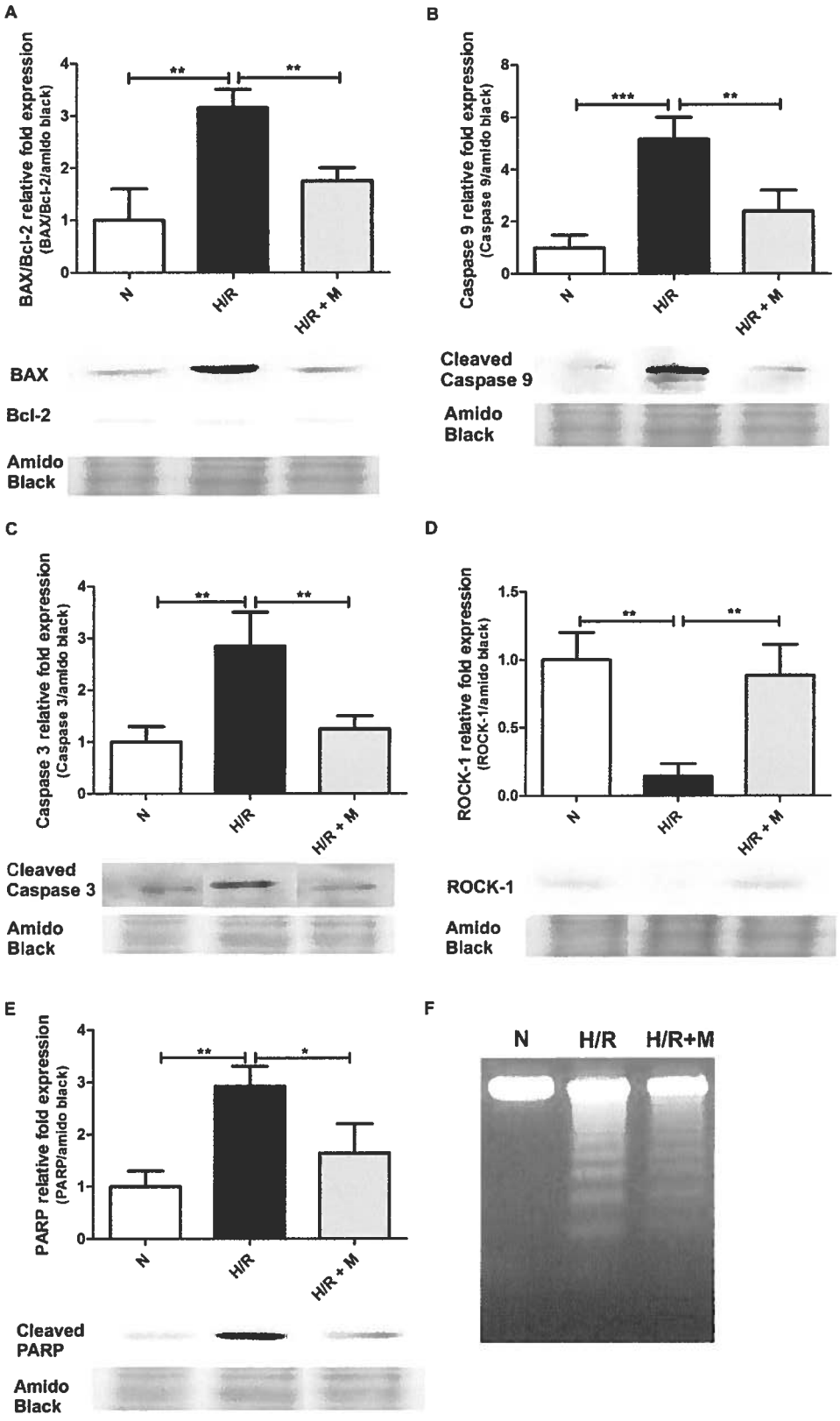

A
BAX/Bcl-2 relative fold expression
(BAX/Bcl-2/amido black)
N
H/R
H/R + M
BAX
Bcl-2
Amido Black
B
Caspase 9 relative fold expression
(Caspase 9/amido black)
Cleaved Caspase 9
Amido Black
C
Caspase 3 relative fold expression
(Caspase 3/amido black)
Cleaved Caspase 3
Amido Black
D
ROCK-1 relative fold expression
(ROCK-1/amido black)
ROCK-1
Amido Black
E
PARP relative fold expression
(PARP/amido black)
Cleaved PARP
Amido Black
F
N
H/R
H/R+M

Figure 5.

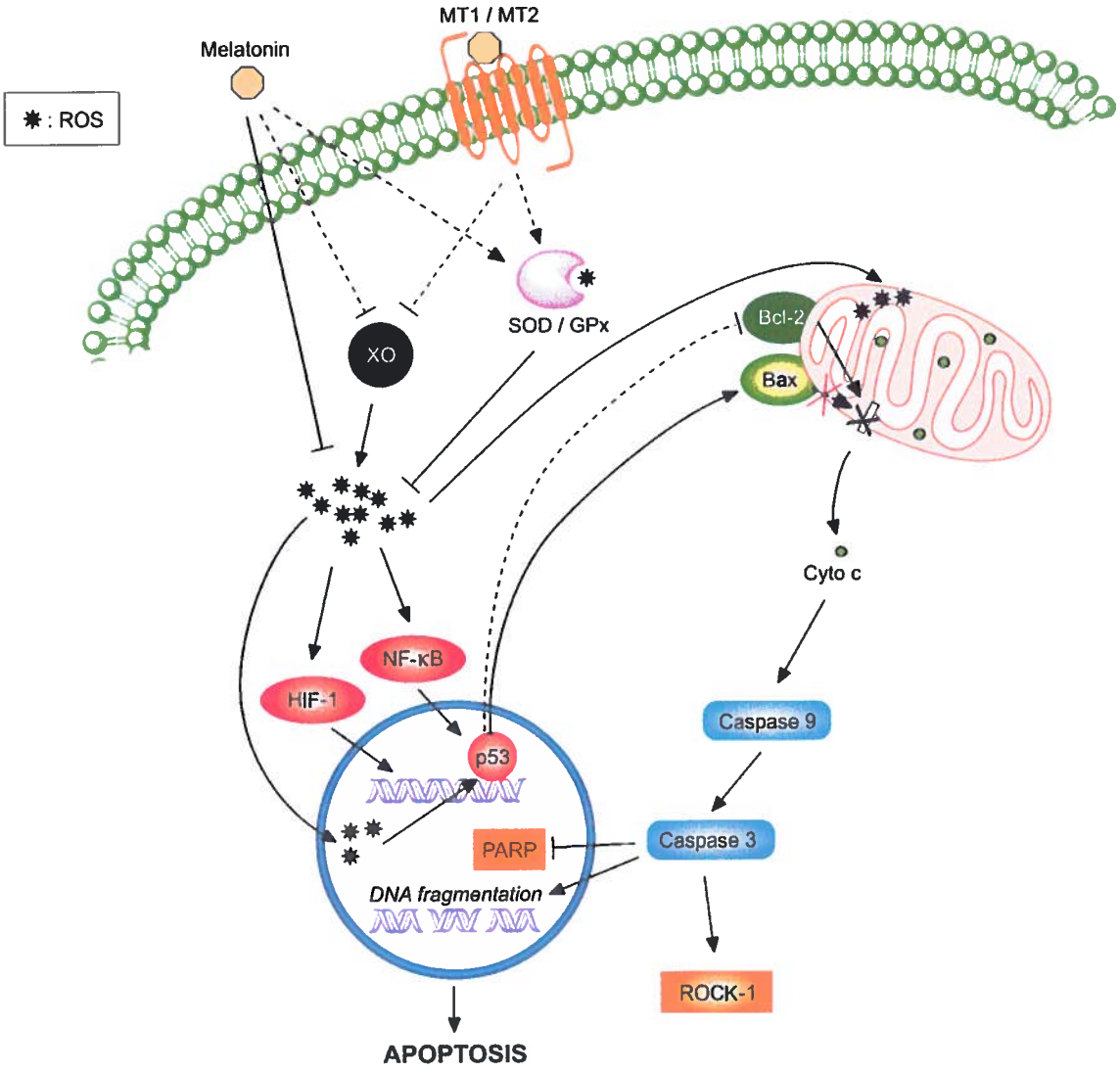

MT1 / MT2
Melatonin
: ROS
SOD / GPx
XO
Bcl-2
Bax
Cyto c
NF-κB
HIF-1
p53
Caspase 9
PARP
Caspase 3
DNA fragmentation
ROCK-1
APOPTOSIS

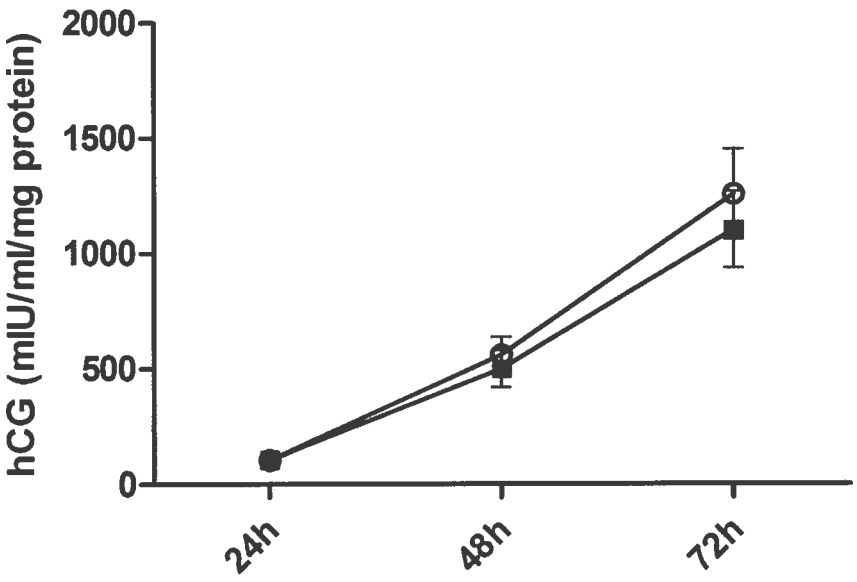
Figure 6.

SUPPLEMENTAL MATERIAL: FIGURE LEGENDS

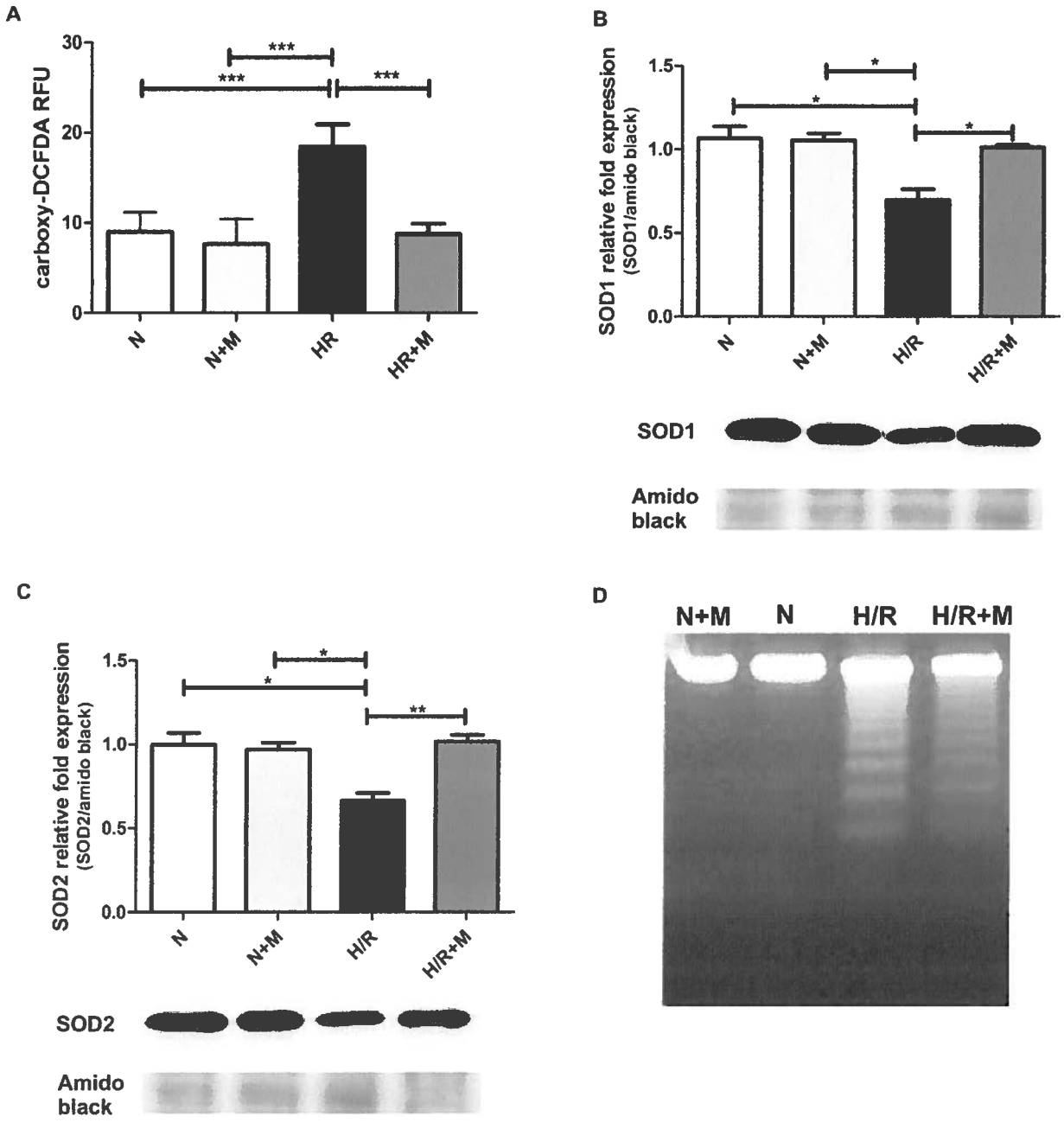
Supplemental data 1. Melatonin does not alter hCG release during villous trophoblast differentiation. Relative hCG release normalized to protein content in primary villous trophoblasts from 0 to 72 h of culture (up to the induction of H/R). hCG release was determined using an hCG enzyme-linked immunosorbent (ELISA) whereas protein content was determined using the bicinchoninic acid (BCA) protein assay reagent. Melatonin (○□); vehicle (■).

Supplemental data 2. Melatonin does not alter cell parameters required to allow villous trophoblast differentiation. **(A)** Relative ROS levels in primary villous trophoblasts were determined using the 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) fluorometric assay. **(B)** SOD1 and **(C)** SOD2 was determined using semi-quantitative Western blot. Amido black stain was used to normalize protein expression. **(D)** DNA fragmentation was evaluated by agarose gel electrophoresis. The different techniques are described in Material and Methods. RFU: relative fluorescence units. N: normoxia (8% O_2, 5% CO_2, 87% N_2); H/R: hypoxia/reoxygenation (0.5% O_2, 5% CO_2, 94.5% N_2 → 8% O_2, 5% CO_2, 87% N_2); 1 mM melatonin. Results are expressed as mean ± SD; n= 3 different placentas; *** $P \leq 0.001$.

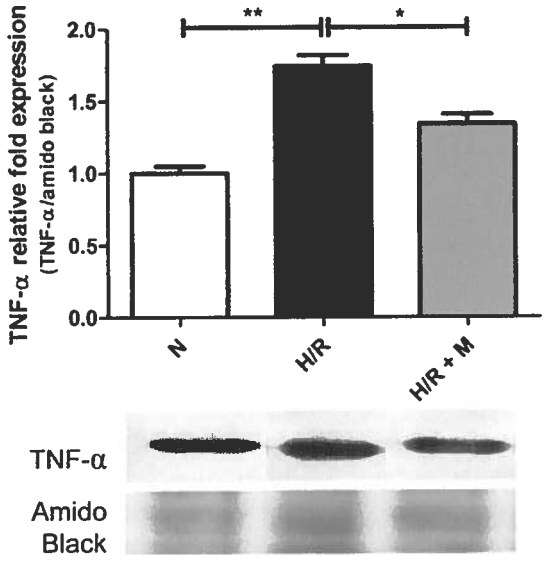
Supplemental data 3. Melatonin prevents the production of TNF-α induced by hypoxia/reoxygenation (H/R). Protein expression of TNF-α was determined using semi-quantitative Western blot as described in Material and Methods. Amido black stain was used to normalize protein expression. TNF-α: tumor necrosis factor α; N: normoxia (8% O_2, 5% CO_2, 87% N_2); H/R: hypoxia/reoxygenation (0.5% O_2, 5% CO_2, 94.5% N_2 → 8% O_2, 5% CO_2, 87% N_2); 1 mM melatonin. Results are expressed as mean ± SD; n=3 different placentas; *** $P \leq 0.001$.



Supplemental data 1



Supplemental data 2.



Supplemental data 3.

CHAPITRE 3: PLACENT AL MELATONIN PRODUCTION AND RECEPTOR EXPRESSION ARE AL TERED IN PREECLAMPSIA: NEW INSIGHT ON THE ROLE OF THIS HORMONE IN PREGNANCY

Dave Lanoix, Pascale Guérin, et Cathy V aillancourt

J Pineal Res. 2012, 53:417-425

PLACENTAL MELATONIN PRODUCTION AND RECEPTOR EXPRESSION ARE AL TE RED IN PREECLAMPSIA: NEW INSIGHT ON THE ROLE OF THIS HORMONE IN PREGNANCY

Résumé de l'article en français

Le système mélatoninergique dans les grossesses prééclampsiques a été grandement négligé, particulièrement au niveau placentaire. Nous avons précédemment démontré la production de mélatonine et l'expression de ses récepteurs MT1 et MT2 dans les placentas humains normaux. De plus, nous et d'autres équipes avons montré le rôle bénéfique de la mélatonine dans les fonctions placentaires et foetal, Indiquant que la mélatonine peut être altérée dans les grossesses pathologiques. Aussi, une diminution des taux sanguins maternels de mélatonine a été montrée dans les cas de prééclampsie comparativement aux grossesses normotensive. Toutefois, les niveaux de mélatonine et l'expression de ses récepteurs dans les placentas de grossesse compliquée par la prééclampsie par rapport aux grossesses normotensive n'ont jamais été étudiés. Conséquemment, dans la présente étude nous avons investigué *:* 1) l'activité et l'expression des enzymes de la synthèse de la mélatonine, 2) la production de la mélatonine et de la sérotonine, précurseur immédiat de la mélatonine, 3) l'expression des récepteurs de la mélatonine dans les placentas compliqués par la prééclampsie et ceux de grossesses normotendues. L'expression de l'ARN et des protéines des enzymes de synthèse de la mélatonine AANAT et HIOMT, les deux enzymes qui convertissent la sérotonine en mélatonine, et de les récepteurs MT1 et MT2 de la mélatonlne ont été déterminé par RTqPCR et par immunobuvardage de type western semi-quantitatif, respectivement. L'activité des enzymes de synthèse de la mélatonine e été déterminé par essai radiométrique, tandis que les niveaux de mélatonine ont été analysés par LC-MS/MS. Les résultats montrent une inhibition significative de AANAT, l'enzyme limitante de la mélatonine, au niveau de l'expression de l'ARN et des protéines et de l'activité dans les placentas prééclampsiques, en corrélation avec une diminution des niveaux de mélatonine. De plus, l'expression de l'ARN et des protéines des récepteurs de la mélatonine MT1 et MT2 est significativement diminuée dans les placentas prééclampsiques comparativement à ceux de grossesses normotensives. Nous proposons que la diminution des taux sanguins maternels de mélatonine puisse être utilisée comme outil de diagnostic précoce pour l'identification de grossesse compliquée par la prééclampsie. Cette étude renforce l'utilité clinique de la mélatonine pour un potentiel traitement de la prééclampsie chez les femmes ou une diminution des taux sanguins a été identifiée.

Contribution de l'étudiant

L'étudiant a réalisé toutes les expériences présentées dans cet article, analysé les résultats, a participé à la rédaction de l'article, au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

Lanoix D, Guérin P, Vaillancourt C. Placental melatonin production and melatonin receptor expression are altered in preeclampsia: new insights into the role of this hormone in pregnancy.
J Pineal Res. 2012 Nov;53(4):417-25.
doi:10.1111/j.1600-079X.2012.01012.x. Epub 2012 Jun 11. PubMed PMID: 22686298.

CHAPITRE 3.1: STABILITY OF REFERENCE PROTEINS IN HUMAN PLACENTA: GENERAL PROTEIN STAINS ARE THE BENCHMARK

Dave Lanoix, Joey St-Pierre, Andrée-Anne Lacasse, Mélanie Viau, Julie Lafond et Cathy Vaillancourt

Placenta. 2012, 33:151-156.

Lanoix D, St-Pierre J, Lacasse AA, Viau M, Lafond J, Vaillancourt C. Stability of reference proteins in human placenta: general protein stains are the benchmark. Placenta. 2012 Mar;33(3):151-6.
doi: 10.1016/j.placenta.2011.12.008.
Epub 2012 Jan 13. PubMed PMID: 22244735.

STABILITY OF REFERENCE PROTEINS IN HUMAN PLACENTA: GENERAL PROTEIN STAINS ARE THE BENCHMARK

Résumé de l'article en français

La stabilité des protéines de références dans les immunobuvardage de type Western semi-quantitatif dans les placentas normaux et pathologiques n'a jamais été étudiée. Cette étude vise à déterminer la stabilité de cinq protéines de référence et de deux types de colorants de protéines dans les placentas de grossesses compliqués par la prééclampsie, le diabète gestationnel mellitus en comparaison à la grossesse normale. La stabilité des protéines de référence a été analysée en utilisant des indicateurs de variabilité inter-groupe (valueur P) et intra-groupe (coefficient de variation). L'effet des différentes stratégies de normalisation a été déterminé en normalisant l'expression du transporteur de la sérotonine (SERT) par rapport à différents marqueurs protéiques. Les résultats montrent une variation significative de l'expression de la 13-actine, la glycéraldéhyde 3-phosphate déshydrogénase (GADPH), l'hypoxanthine phosphoribosyltransférase-1 (HPRT1), la peptidylprolyl isomérase A (PPIA) et l'a-tubuline, alors que la coloration avec amido black est le marqueur protéique le plus stable. De plus, les résultats montre que l'expression de SERT est significativement différente selon le marqueur protéique utilisé pour la normalisation. Cette étude démontre l'importance d'utiliser des marqueurs protéiques de référence stable afin d'obtenir des résultats valides dans les analyses d'immunobuvardage de type western semi-quantitatif avec les tissus placentaires.

Contribution de l'étudiant

L'étudiant a réalisé la plupart des expériences présentées dans cet article. Il a choisi le design expérimental, analysé les résultats, participé à la rédaction de l'article, au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

CHAPITRE 3.2: QUANTITATIVE PCR PITFALLS: THE CASE OF THE HUMAN PLACENTA

Dave Lanoix, Andrée-Anne Lacasse, Joey St-Pierre, Sean Taylor, Maude Éthier-Chiasson, Julie Lafond et Cathy Vaillancourt

Molecular Biotechnology. 2012, 52:234-243

QUANTITATIVE PCR PITFALLS: THE CASE OF THE HUMAN PLACENTA

Résumé de l'article en français

La réaction de transcription inverse (RT)-couplée à la réaction en chaîne par polymérase quantitative (qPCR) est une technique de génétique rapide et de haut débit permettant la quantification de l'expression des gènes. Afin d'obtenir des résultats précis, plusieurs étapes de validation doivent être rigoureusement suivies telles que décrites dans les lignes directrices du : *Minimum Information for Publication of Quantitative Real-Time PCR Experiment* (MIQE). Cette étude analyse les effets de la normalisation en utilisant plusieurs gènes de références et l'impact de la dégradation de l'ARN sur l'expression de la 8-oxoguanine ADN glycosidase 1 (OGG1) dans le placenta humain provenant de grossesses compliquées par la prééclampsie et le diabète gestationnel mellitus comparativement à leurs contrôles. Les résultats montrent que la qualité de l'ARN et l'utilisation de gènes de référence appropriés n'est pas seulement importante pour l'obtention de résultats de RT-qPCR valide et reproductible, mais aussi comment des résultats différents et même significativement opposés peuvent être obtenu si les étapes du MIQE ne sont pas respectées. Les méthodes et les résultats présentés dans cette étude fournissent la première application pratique des lignes directrices de la MIQE dans l'analyse de placentas provenant de grossesses pathologiques et normales.

Contribution de l'étudiant

L'étudiant a accompli toutes les expériences, à l'exception des RT-qPCR réalisés avec les échantillons provenant de placentas de grossesses compliquées par le diabète gestationnel mellitus et leurs contrôles, présentés dans cet article. Il a choisi le design expérimental, analysé les résultats, a participé à la rédaction de l'article, au choix du journal de publication et à la publication de la version finale de l'article.

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RESEARCH

Quantitative PCR Pitfalls: The Case of the Human Placenta

Dave Lanoix · Andrée-Anne Lacasse · Joey St-Pierre · Sean C. Taylor · Maude Ethier-Chiasson · Julie Lafond · Cathy Vaillancourt

Abstract Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is a rapid and high throughput gene expression quantification technology. In order to obtain accurate results, several key experimental design and standardization steps must be rigorously followed as previously described in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. This study investigates the effect of reference gene normalization and the impact of RNA degradation on gene expression of 8-oxoguanine DNA glycosylase in human placenta from pregnancies complicated by preeclampsia and gestational diabetes mellitus and their gestation-matched controls. The data presented here show how RNA quality and appropriate reference gene selection is not only important to obtain accurate and reproducible RT-qPCR data but how different and even opposite results can be reported if the key steps outlined in the MIQE guidelines are not followed. The procedures and associated results presented in this study provide the first practical application of the MIQE guidelines to placental analysis in normal and pathological pregnancies.

Keywords Gestational diabetes · Preeclampsia · 8-Oxoguanine DNA glycosylase · Real-time RT-PCR · Reference gene · RNA integrity

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Introduction

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is the method of choice to quantify differences in gene expression levels between messenger RNA (mRNA) samples. It is a highly sensitive technique that requires validation at several steps to assure accurate results. Bustin et al. [1] established the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines to ensure that published articles with qPCR data are accurate and reproducible. Unfortunately, many laboratories still do not follow the MIQE guidelines raising concerns among the scientific community over the reliability of RT-qPCR data and the risk of reporting erroneous and conflicting results [2, 3]. One of the most famous cases is the retraction of a Science "Breakthrough of the year 2005" report [4] for incorrect data analysis. Even more recently and much farther reaching was a retraction from *The Lancet* [5] linking the measles, mumps, and rubella vaccination to autism in children where the results were based mostly on flawed RT-qPCR data and associated interpretation.

Sample handling and validation steps prior to RT-qPCR are crucial and can be a major source of error. For example, degraded RNA can have a large impact on RT-qPCR data and on the associated conclusions [6]. Degradation of RNA may be due to many factors including poor sample

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handling, inappropriate storage or transport conditions [7, 8]. This is particularly the case for tissues which require storage in an RNA preservation solution or snap frozen immediately after extraction to avoid RNA degradation [1]. This is often difficult or impossible with human tissue samples given the variability in surgical procedures and the associated surgical staff in extracting and storing these samples.

The placenta is an accessible human tissue for research since it is a temporary organ. However, at delivery the focus is towards the newborn and if there are any complications associated with birth, placenta processing time can vary widely between patients. Moreover, the human placenta is an organ with high levels of RNAse [9]. These factors combined make this tissue susceptible to wide variability both in RNA degradation and in sample handling which can lead to artifactual changes in gene expression. No studies have assessed the effect of RNA integrity on RT-qPCR results in human placenta.

Housekeeping genes are commonly used to normalize gene expression primarily because they are assumed to be consistently expressed under most experimental conditions. However, in many tissues including the placenta, the gene transcripts of common housekeeping genes are subject to instability and variability [10–12]. Several physiological and pathological processes can influence housekeeping gene expression, such as cellular proliferation and differentiation [13–15] as well as cellular stress and metabolism [16]. Consequently, housekeeping genes are subject to instability and variability between normal and pathologic conditions. It is of particular concern for studies between placentas from normal and preeclamptic or gestational diabetes mellitus pregnancies because these cellular processes play key roles in their pathogenesis [17–20]. Bioinformatics approaches have been developed to uncover appropriate reference genes [21–23]. However, suitable reference gene targets between placentas from preeclamptic, gestational diabetes mellitus and their respective gestation-matched controls have never been studied using the tools and procedures recommended by the MIQE guidelines. This study characterizes the stability of nine commonly used reference genes, 18S ribosomal RNA (rRNA), β-actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), peptidylprolyl isomerase A (PPIA), succinate dehydrogenase complex, subunit A (SDHA), TATA box binding protein (TBP), topoisomerase 1 (TOP1) and tyrosine 3-monooxygenase/tryptophane 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) in placentas from normal, preeclamptic and gestational diabetes mellitus pregnancies. The effect of normalization by these reference genes and of RNA degradation on 8-oxoguanine DNA glycosylase (OGG1) expression in these placentas was also examined.

Methods

Placental Tissues

Placentas from preeclampsia or gestational diabetes mellitus and their respective gestation-matched controls were obtained with informed patient consent and approval of ethical committees at the Centre Hospitalier Universitaire de Montréal (CHUM)-St-Luc Hospital, Montreal, QC. Clinical pathologic characteristics of patients and placentas are summarized in Table 1. Preeclampsia was diagnosed by a clinician according to American Congress of Obstetricians and Gynecologists criteria [24]. Gestational diabetes mellitus was diagnosed according to criteria from American Diabetes Association [25]. Patients with gestational diabetes mellitus were supplemented with insulin.

Table 1 Clinical pathologic characteristics of patients and placentas

Variable	N vs. PE		N vs. GDM	
	N (n = 11)	PE (n = 11)	N (n = 8)	GDM (n = 8)
Maternal age (years)	28.4 ± 8.3	29.0 ± 3.8	29.83 ± 4.4	31.67 ± 6.6
BMI (kg/m^2)	N/A	N/A	22.56 ± 2.97	28.46 ± 6.65[a]
Gestational age (weeks)	36.1 ± 4.3	33.1 ± 4.7	40.13 ± 1.8	39.4 ± 0.7
Mode of delivery (V:C)	7:4	5:5	5:3	4:4
Newborn sex (F:M)	6:5	8:3	3:5	4:4
Newborn weight (g)	2686 ± 663.5	2212 ± 999.9	3541 ± 373.3	3443 ± 376.5
Placenta weight (g)	456.6 ± 165.2	291.6 ± 130.4	641.7 ± 127.1	646.9 ± 192.0

The same cohort as our previous study was used [33]

BMI body mass index, *C* caesarean section, *F* female, *M* male, *V* vaginal, *N* normotensive or non-gestational diabetes mellitus, *PE* preeclampsia, *GDM* gestational diabetes mellitus

[a] $P = 0.001$

Women having pathologies (other than preeclampsia or gestational diabetes mellitus), smokers or under medication (other than insulin for gestational diabetes mellitus) were excluded.

All samples were processed within 30 min of placental delivery. Placental tissue samples were excised from randomly selected regions according to Mayhew [26]. Decidua and amnion were removed, placental tissue was cut in small pieces, thoroughly rinsed in physiological saline (0.9 % NaCl), snap-frozen in liquid nitrogen and stored at −80°C.

RNA Extraction

Total RNA was extracted from frozen placental tissues using the Aurum Total RNA mini kit (Bio-Rad, Mississauga, Canada) according to manufacturer's Spin Protocol for Animal Tissue. Briefly, placental tissue was ground into a fine powder with a mortar and pestle containing liquid nitrogen. 15 mg of tissue was transferred to an RNase-free microcentrifuge tube pre-cooled into liquid nitrogen. The lysis solution was added without letting the tissue thaw and was thoroughly vortex. From this point, all steps were performed at room temperature. The lysate was transferred to a QIAshredder spin column homogenizer (Qiagen, Toronto, Canada) and centrifuged for 2 min at 20,000×*g* to completely disrupt the tissue. The disrupted tissue was then thoroughly mixed with 60% ethanol (1:1) and transferred to the RNA binding column and processed according to manufacturer's instructions. Genomic DNA contamination was removed by performing DNase I digestion on the RNA binding column for 25 min at RT. Total RNA was eluted in 80 µl of 70°C preheated elution buffer and stored at −80°C.

RNA Quality Control

RNA quantity and purity was assessed spectrophotometrically by measuring the OD_{260} and $OD_{260/280}$ ratio respectively in TE buffer (10 mM Tris, pH 7.0, 1 mM EDTA) using a SpectraMax M5 microplate reader (Molecular Devices, Toronto, Canada). Samples were considered for further analysis when the $OD_{260/280}$ ratio was between 1.8 and 2.0.

RNA integrity was determined using the Experion™ Automated Electrophoresis Station with the standard-sensitivity RNA analysis kit according to manufacturer's instructions (Bio-Rad). The electropherogram and virtual gel were evaluated using the Experion system's software that generated an RNA quality indicator (RQI). The RQI values ranged from 1 (highly degraded RNA) to 10 (intact RNA). RNA samples were considered intact when the RQI value was over 8. Samples with RQI values between 3 and 5 were chosen for the experiment using degraded RNA.

First Strand Complementary DNA (cDNA) Synthesis

RNA samples that successfully met the quality control standards were reverse transcribed to cDNA with the iScript cDNA synthesis kit using a blend of oligo(dT) and random hexamer primers according to manufacturer's instructions (Bio-Rad). In 20 µl reactions, 750 ng of total RNA was incubated with the reaction mix, reverse transcriptase and nuclease-free water for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. No reverse transcriptase (NRT) controls were prepared for each sample. First strand cDNA samples were stored at −20°C.

Quantitative PCR (qPCR)

Primers were designed using Oligo 6 software (Molecular Biology Insights, Cascade, USA) and their specificity was determined with Primer-Blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Amplicons were tested for potential secondary structure using mfold web server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) [27]. Primer specificity was confirmed by agarose gel electrophoresis after RT-qPCR reaction. Validated sequences are shown in Table 2.

Standard curves for each target were prepared from pooled samples of first-strand cDNA from preeclamptic, gestational diabetes mellitus placentas, and their respective gestation-matched controls. Four randomly selected cDNA samples from each group were pooled and 8 serial dilutions were made. Reaction efficiencies were determined from the standard curves.

SsoFast EvaGreen Supermix (Bio-Rad) was used for amplification of selected genes. Reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad). Briefly, the cycle conditions were as follows: 30 s at 95°C for enzyme activation, 40 cycles of denaturation at 95°C for 5 s and combined annealing/extension at 60°C for 5 s followed by a melting curve ranging from 65°C to 95°C by steps of 0.5°C for 5 s. No template control (NTC) and NRT reactions were performed to assure no foreign or genomic DNA contamination during sample and master mix preparation.

Data Analysis

Statistical analysis was performed using Prism (GraphPad, La Jolla, USA). P values <0.05 were considered statistically significant. The stability of candidate reference genes was analyzed using geNorm, version 3.5 [22], and NormFinder [21] software. geNorm is a VBA applet for Microsoft Excel freely available on the internet: http://medgen.ugent.be/~jvdesomp/genorm/. The software calculates the

Table 2 Genes and primer sequences used for RT-qPCR

Gene	Accession number	Primer sequence	
		Sense	Antisense
18S	X03205	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
ACTB	NM_001101	AAACTACCTTCAACTCCATC	ATGATCTTGATCTTCATTGT
GAPDH	NM_002046	GAAGGTGAAGGTCGGAGTCAA	GGAAGATGGTGATGGGATTTC
HPRT1	NM_000194	GACCAGTCAACAGGGGACATAA	AAGCTTGCGACCTTGACC
hOGG1	NM_002542	TGGAAGAACAGGGCGGGCTA	ATGGACATCCACGGGCACAG
PPIA	NM_021130	GTTTGCAGACAAGGTCCCA	ACCCGTATGCTTTAGGATG
SDHA	NM_004168	TACAAGGTGCGGATTGATG	CGATCACGGGTCTATATTCAA
TBP	NM_003194	CACGAACCACGGCACTGAT	GTTGGTGGGTGAGCACAAGG
TOP1	NM_003286	GGCAGAGTGAATCTAAGG	CTTAAAGGGTACAGGAATG
YWHAZ	NM_003406	GGCAACCTAAGAACAAATG	CATGTTAGGCAAGTATCAAA

gene expression stability "M" value for each candidate reference gene and ranks them accordingly from low to high [22]. geNorm was also used to determine the optimal number of reference genes required for normalization by calculating the pairwise variation V between two sequential normalizations containing an increasing number of genes. A cut-off value of 0.15 was established below which an additional reference gene is not required [22].

NormFinder is a Microsoft Excel add-in freely available on the internet: http://www.mdl.dk/publicationsnormfinder.htm. The NormFinder algorithm estimates overall gene expression variation as well as the variation between subgroups, such as normal and diseased placental samples [21]. A low stability value indicates high intra- and inter-group expression stability.

Results

RNA Quality Assessment

The quantity and purity of RNA samples from normal and diseased placentas was monitored spectrophotometrically. Samples with an $OD_{260/280}$ ratio between 1.8 and 2.0 were analyzed with the Experion system (Fig. 1). Although there has been no accepted threshold RQI number for acceptable RNA samples, studies have shown high expression variability for RQI below 7 and even below 7.8 [28–30]. Therefore in this work an RQI value above 8.0 was chosen for intact samples. A representative electropherogram of an intact RNA sample, showing the 18S and 28S rRNA peaks, is presented in the Fig. 1a. Samples with an RQI value between 3.0 and 5.0 were considered degraded and the associated electropherograms show multiple peaks with the 18S and 28S rRNA (Fig. 1c). Figure 1c shows the virtual gel images of three representative intact and degraded RNA samples.

Expression Levels of Reference Genes in Normal and Diseased Placentas

The relative expression levels of nine candidate reference genes were analyzed in placentas from pregnancies complicated by preeclampsia and gestational diabetes mellitus as well as their gestation-matched controls (Fig. 2a, b). In normotensive versus preeclamptic pregnancies placentas, quantitative cycle (C_q) values ranged from 19.43 to 31.9 while in non-gestational diabetes mellitus vs gestational diabetes mellitus placentas, they spanned from 18.01 to 32.03. The highest expression level, i.e. the lowest C_q value, in both normotensive versus preeclamptic and non-gestational diabetes mellitus vs gestational diabetes mellitus pregnancies placentas is 18S rRNA whereas the gene with the lowest expression level is TBP.

Expression Stability of Reference Genes

An ideal reference gene displays a similar expression level within and between each sample group analyzed. To determine the most stable reference genes, the expression stabilities of the nine candidate targets were analyzed using the geNorm software tool [22]. The MIQE Guidelines define reference gene stability with M values below a threshold of 1.0 for heterogeneous samples and below 0.5 for homogeneous samples. The average M value of each gene in normotensive and preeclamptic pregnancies placentas is displayed in Fig. 3a. The least stable gene with an M value of 1.20 is 18S rRNA. The M value of all the other genes is between 0.93 and 0.51. HPRT1 and PPIA were the two most stably expressed genes. To determine the optimal number of reference genes to use, a pairwise variation analysis with geNorm V was performed. In placentas from normotensive vs preeclamptic pregnancies, the geometric mean of three genes displays a higher stability than with just two genes with a pairwise variation of 0.148 versus

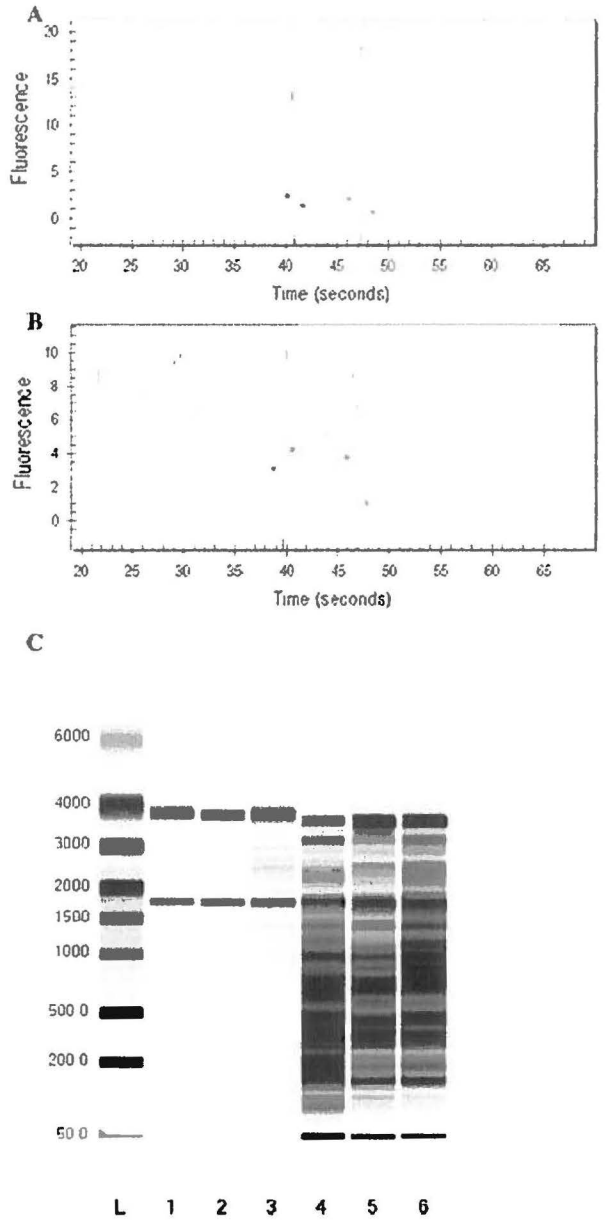


Fig. 1 Analysis of RNA intregity. Human term placenta total RNA samples were analyzed for integrity using the Experion™ Automated Electrophoresis Station. Representative electropherogram of microfluidic capillary electrophoresis for (**a**) intact and (**b**) degraded RNA samples. (**c**) Virtual gel generated by the Experion™ Automated Electrophoresis Station showing intact (*lanes* 1–3) and degraded (*lanes* 4–6) RNA samples. *L* ladder (base pair)

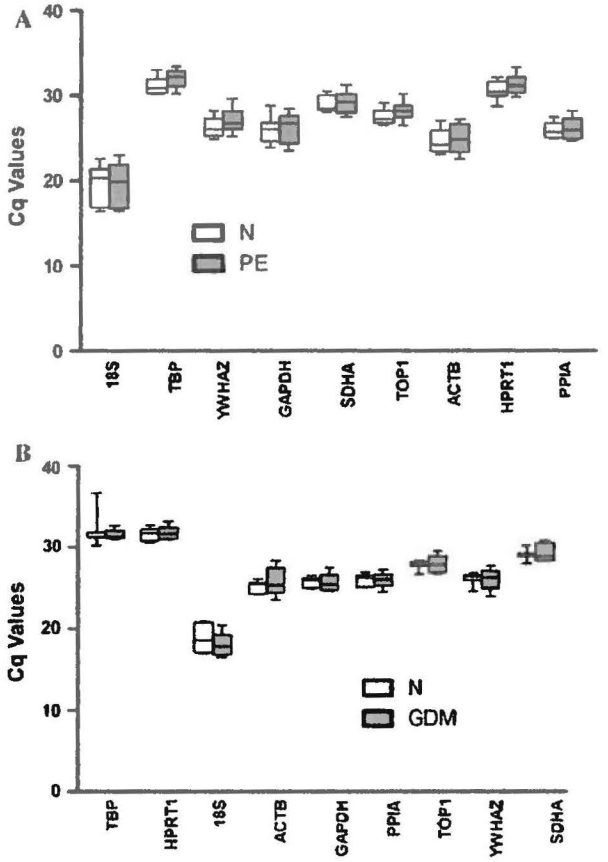
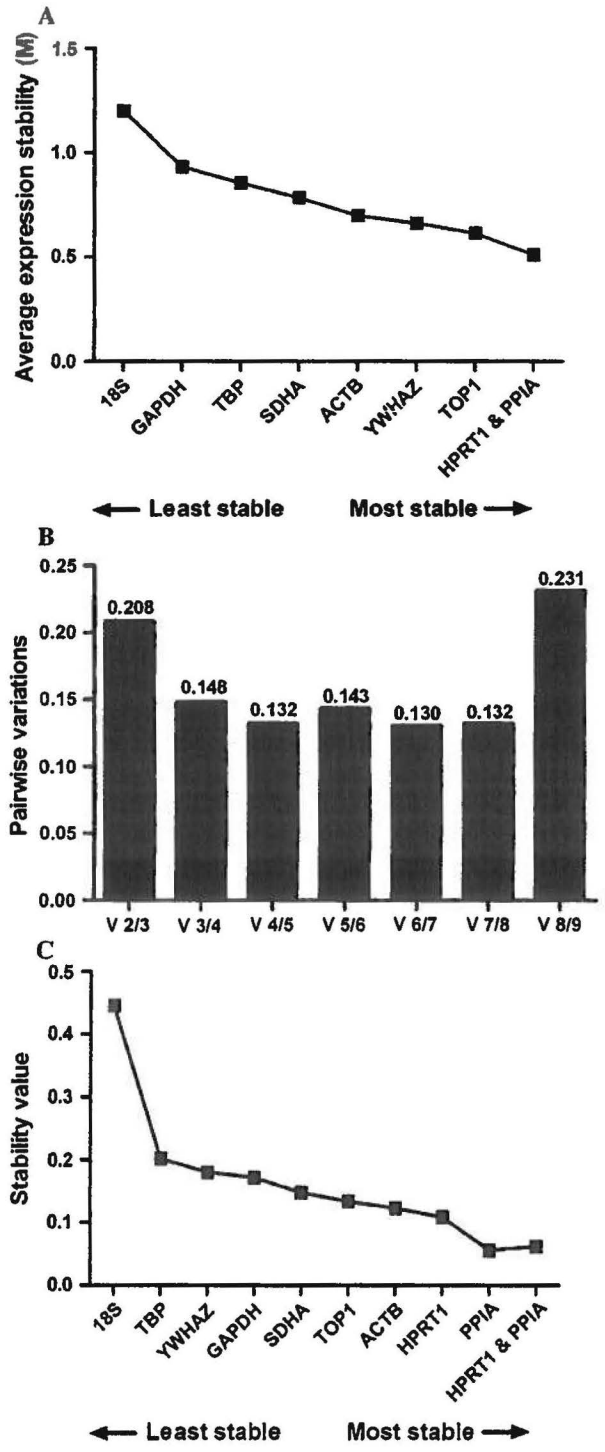


Fig. 2 Expression level of reference genes. Expression of 9 reference genes in placentas from (**a**) preeclamptic (PE) and (**b**) gestational diabetes mellitus (GDM) pregnancies and their respective gestation-matched controls (N). Values represent the real-time PCR cycle threshold numbers (C_q values). Boxes represent the lower and upper quartiles with medians

0.208 (Fig. 3b). Thus, TOP1, HPRT1, and PPIA were used to normalize gene expression between normotensive and preeclamptic pregnancies placentas. NormFinder was also used to examine reference gene stability and gave similar results to geNorm with HPRT1 and PPIA giving the highest stability followed by ACTB and TOP1 (Fig. 3c). Interestingly YWHAZ was ranked with low stability in the reference gene set using NormFinder as opposed to geNorm which ranked this gene in the top four most stable. Hence, YWHAZ was treated as a questionable reference gene given the inconsistent ranking using the two software tools.

The average M value in non-gestational diabetes mellitus vs gestational diabetes mellitus pregnancies placentas is shown in Fig. 4a. The least stable gene is 18S rRNA with an M value of 1.94. The M value of all other genes in gestational diabetes mellitus pregnancies was between 0.98 and 0.46 with the two most stable identified as PPIA and YWHAZ. The geometric mean of three genes between these two groups gave a higher stability than with two with a pairwise variation of 0.104 (Fig. 4b). Therefore, the reference genes used in non-gestational diabetes mellitus and gestational diabetes mellitus pregnancies placentas were GAPDH, PPIA and YWHAZ. Using NormFinder, the three most stable genes in non-gestational diabetes mellitus



◀ **Fig. 3** Analysis of reference genes stability in placentas from normotensive and preeclamptic pregnancies. (a) geNorm M analysis was performed to determine reference gene expression stability. The lowest M value characterized the most stably expressed genes. (b) The optimal number of reference genes to be used determined by geNorm V analysis. Pairwise variations ($V_{n/n+1}$) determine the least number of reference genes necessary for an accurate normalization. (c) NormFinder program was used to determine reference gene stability based on an estimate of intra- and inter-group variability. *N* normotensive pregnancies placentas, *PE* preeclamptic pregnancies placentas

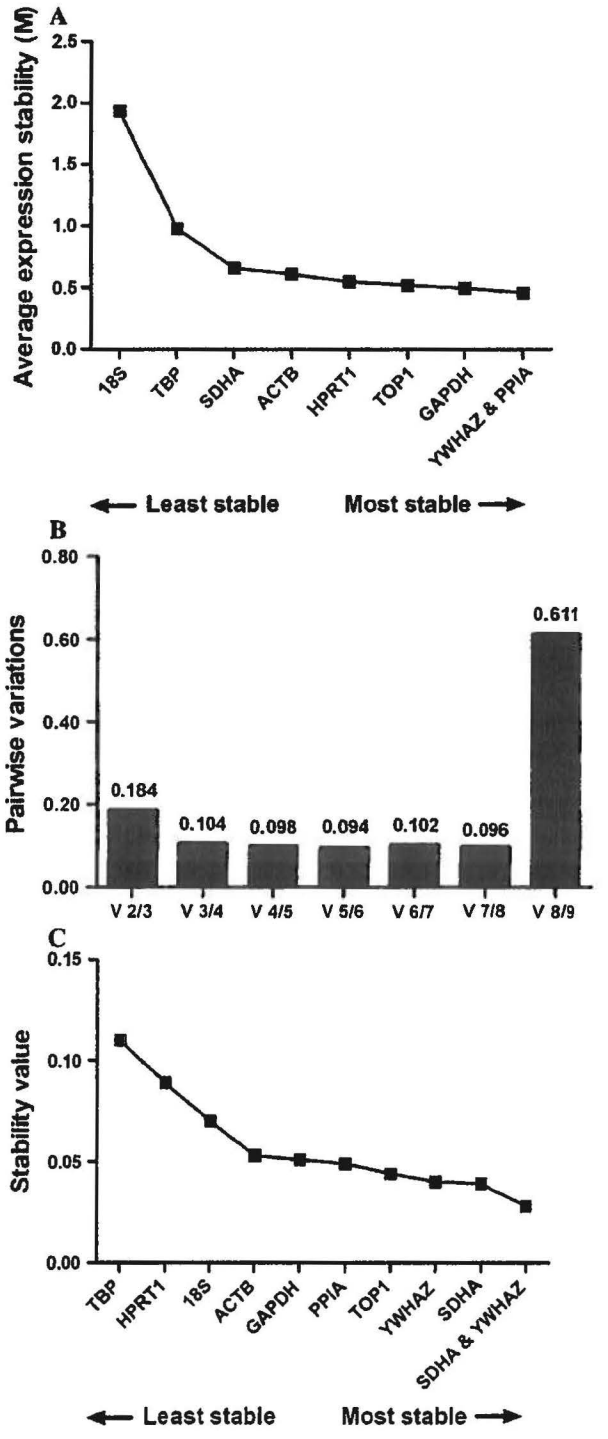
vs gestational diabetes mellitus pregnancies placentas are SDHA, YWHAZ and TOP1 with PPIA and GAPDH ranked the fourth and fifth most stable genes (Fig. 4c). In this case, SDHA was ranked with very high stability in the reference gene set using NormFinder as opposed to geNorm which ranked this gene in the bottom three least stable. Hence, SDHA was treated as a questionable reference gene given the inconsistent ranking using the two software tools.

Effect of Reference Gene Normalization on Gene Expression

To demonstrate the effect of reference gene normalization on gene expression data, the target gene OGG1 was chosen as a good positive control with known and well-characterized activity in DNA repair that is induced by oxidative stress, such as in preeclamptic pregnancies placentas [6]. Normalizing OGG1 expression with the nine individual reference genes and the combination of the 2 or 3 most stable reference genes gave contrasting results (Fig. 5a). As predicted, a significant increase of OGG1 expression was observed in preeclamptic relative to normotensive pregnancies placentas when normalized to the 3 most stable genes, PPIA, HPRT1, and TOP1 either individually or in combination. However, normalization using 18S rRNA gave the opposite results where OGG1 is significantly decreased in preeclamptic pregnancies placentas. In addition, normalization with YWHAZ, SDHA, GAPDH or TBP did not show any significant difference in OGG1 expression. The expression of OGG1 in gestational diabetes mellitus pregnancies placentas was also analyzed and the associated normalization with the different reference genes also gave contrasting results (Fig. 5b). A non-significant inhibition was observed with OGG1 expression when normalized to the three most stable genes, YWHAZ, PPIA and GAPDH as well as with TOP1 and HPRT1. However, using ACTB and SDHA, a significant increase was noted while with 18S rRNA normalization a significant inhibition of OGG1 expression was observed.

Effect of RNA Sample Degradation on Gene Expression

To demonstrate the importance of RNA quality control, placentas from three normotensive and three preeclamptic pregnancies with degraded RNA were pooled with the 11



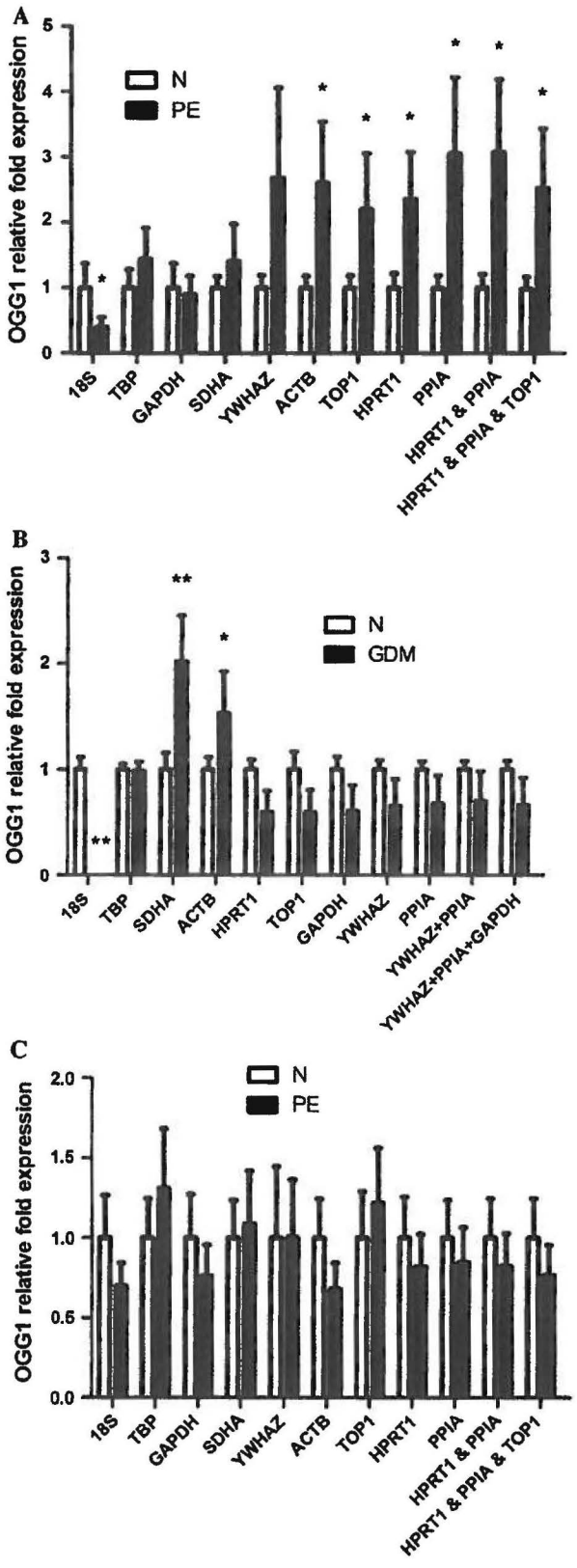
◀ **Fig. 4** Analysis of reference gene stability in placentas from non-gestational diabetes mellitus pregnancies. **(a)** geNorm M analysis was performed to determine reference genes expression stability. The lowest M value correlates to the most stably expressed gene. **(b)** The optimal number of reference genes to be used determined by geNorm V analysis. Pairwise variations ($V_{n/n+1}$) determine the least number of reference genes necessary for an accurate normalization. **(c)** NormFinder program was used to determine reference gene stability based on an estimate of intra- and inter-group variability. *N* non-gestational diabetes mellitus pregnancies placentas, *GDM* gestational diabetes mellitus pregnancies placentas

intact RNA samples from each group respectively resulting in complete abrogation of the significant differences observed with intact samples (Fig. 5c). In addition, the relative expression of OGG1 is lower when the degraded RNA samples are included.

Discussion

This study demonstrates, for the first time, the importance of using intact RNA samples and the rigorous selection of stable reference genes to assure accurate RT-qPCR results with placentas from pregnancies complicated by pre-eclampsia and gestational diabetes mellitus as well as their gestation-matched controls. These results conformed to the predicted and previously observed relative difference of OGG1 expression between the preeclamptic sample sets [31]. In addition, these are the first results to show that OGG1 placental expression was not affected by gestational diabetes mellitus and confirms that to ensure the accuracy of RT-qPCR data, it is essential to design experiments that conform to the MIQE guidelines [1, 32]. In fact, our recent study demonstrating significantly altered expression differences for target proteins in placentas from preeclamptic and gestational diabetes mellitus complicated pregnancies when normalized to inappropriate loading control proteins shows that similar guidelines should be applied to western blotting [33].

As described in the MIQE guidelines, normalization of target genes requires stably expressed reference genes between the samples from all the test conditions [1]. The first and currently the most popular software tool to determine reference gene stability is geNorm [22]. Other programs have been developed to identify reference genes, such as NormFinder [21]. Therefore to validate our results, we used both software tools and found a good correlation for reference gene stability between placentas from normotensive and preeclamptic pregnancies with the exception of YWHAZ which was ranked in the top four most stable with geNorm and the bottom three with NormFinder (Fig. 3). When reference gene stability was tested between gestational diabetes mellitus pregnancies placentas there was again good correlation between the two software tools with the exception of SDHA which was ranked with the second highest stability using NormFinder as opposed to the geNorm ranking as the third least stable (Fig. 4). The combined data from geNorm and NormFinder permitted appropriate selection of reference genes and the exclusion of questionable outliers.



◀**Fig. 5** Effect of reference gene normalization and RNA degradation on hOGG1 expression in normal and pathologic pregnancies placentas. Variability of hOGG1 expression normalized against the tested reference genes and the best combinations in (**a**) 11 normotensive and 11 preeclamptic as well as (**b**) 8 non-gestational diabetes mellitus and 8 gestational diabetes mellitus pregnancies placentas with intact RNA samples. (**c**) Effect of RNA degradation, 3 degraded RNA samples from normotensive and preeclampsia pregnancies placentas were pooled with 11 intact RNA samples of each group respectively. *N* normotensive of non-gestational diabetes mellitus pregnancy; *PE* preeclamptic pregnancy; *GDM* gestational diabetes mellitus pregnancy. Mean ± SD. $*P < 0.05$; $**P < 0.01$

In the current literature, no study has analyzed reference gene expression stability in either normotensive vs preeclamptic as well as non-gestational diabetes mellitus vs gestational diabetes mellitus pregnancies placentas. To illustrate the significance of selecting appropriate reference genes to normalize target gene expression, the normalized expression of the well-characterized target OGG1 was analyzed in normotensive versus preeclamptic pregnancies placentas. The geometric mean of the three most stable reference genes exhibited a significant increase of OGG1 expression in preeclamptic compared to normotensive pregnancies placentas. This is in accordance with the current literature [31]. However, normalization with inappropriate reference genes such as 18S rRNA can result in statistically significant opposite results than predicted. Furthermore normalization with other reference genes can result in no significant difference in OGG1 expression which is again contrary to the published data.

Murthi et al. [11] determined the stability of 6 reference genes between normal and fetal growth restriction pregnancies placentas and concluded that GAPDH, 18S rRNA and YWHAZ were the most suitable reference genes. Our data support the work from Meller and colleagues who assert that 18S rRNA is not a suitable reference gene in human placenta [10, 34]. However, they conclude that TBP, SDHA and YWHAZ had the greatest stability and therefore, should be used in placenta qPCR studies regardless of the test group. Our results demonstrate that reference gene stability must be analyzed between the tested experimental conditions for a given experiment and in this study resulted in a completely different ranking of reference gene stability between the two disease groups. These contrasting results are not uncommon in the literature of papers published with qPCR data and support the need for experimental design guidelines such as those described by the MIQE criteria.

The integrity of RNA can also significantly impact gene expression studies [6]. Since about 20% of placenta tissue RNA samples were significantly degraded in the present study as shown by the RNA integrity method used here, the

effect of RNA degradation on OGG1 expression was tested. As expected, OGG1 expression with degraded RNA is reduced compared to intact RNA samples and the significant difference in normalized expression between normotensive and preeclamptic pregnancies placentas is lost.

In conclusion, the framework provided by MIQE Guidelines to assure quality data for RT-qPCR was applied in this study to give the expected regulation in gene expression for OGG1. The high propensity for variability in the handling of human placenta tissue underlines the importance of rigorously testing the extracted RNA for purity and integrity. Moreover, it is crucial to analyze reference gene stability between the tested experimental conditions to avoid producing artifactual data and the use of both geNorm and NormFinder helps assure the selection of appropriate reference genes for a given study. These data have widespread implications on the analysis of the many types of human tissue that are sensitive to degradation for which sample handling and storage is inconsistent. In the cases observed here, RT-qPCR expression data can literally shift from positive to negative to no regulation simply by ignoring key validation steps from the MIQE guidelines such as RNA quality and appropriate reference gene selection.

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DISCUSSION ET CONCLUSIONS GÉNÉRALES

La présente étude a permis de caractériser la mélatonine placentaire (production et expression de ses récepteurs), de déterminer si elle pouvait préserver l'homéostasie du trophoblaste villeux, qui est notamment perturbée dans les placentas de prééclampsie, et de comparer la mélatonine (production et expression de ses récepteurs) entre des placentas de grossesses normotensives et compliquées par la prééclampsie.

Les résultats démontrent : 1) la production de mélatonine et l'expression de ses récepteurs MT1 et MT2 dans des cultures primaires de trophoblaste villeux isolé de placentas humain normal à terme; 2) l'action protectrice de la mélatonine contre le l'augmentation de l'apoptose mitochondriale du syncytiotrophoblaste induit par une H/R et 3) une diminution significative de la production de mélatonine et de l'expression de ses récepteurs MT1 et MT2 dans les placentas de grossesses compliqués par la prééclampsie en comparaison à la grossesse normale.

Premièrement, mes travaux de doctorat ont permis de démontrer que le placenta humain normal à terme produit *de novo* la mélatonine (**Chapitre 1**). Les enzymes AANAT et HIOMT, assurant la synthèse de la mélatonine sont exprimées et actives dans le placenta. Des analyses réalisées sur des cultures primaires de cellules du trophoblaste villeux indiquent que celles-ci, en particulier le syncytiotrophoblaste, sont responsables de la majorité de la production placentaire de mélatonine à terme. L'activité de l'enzyme AANAT, limitante pour la synthèse de la mélatonine, dans les cytotrophoblastes villeux et le syncytiotrophoblaste est en moyenne trois et cinq fois plus élevée que dans la glande pinéale durant la nuit respectivement (Ackermann *et al.*, 2006). Considérant la masse du placenta à terme en comparaison à celle de la glande pinéale (Castellucci *et al.*, 2006, Reichlin, 1998), on estime qu'il produit jusqu'à 10 000 fois plus de mélatonine (Lanoix *et al.*, 2008). Les taux sanguins de mélatonine durant la grossesse sont augmentés tant durant le jour que la nuit (Kivela, 1991, Nakamura *et al.*, 2001), suggérant une

source de production de mélatonine non circadienne. Les concentrations élevées en mélatonine mesurées dans le tissu placentaire par Nakazawa et ses collègues ne subissent également pas de variations circadiennes (Nakazawa *et al.*, 1999). De plus, aucun effet de la grossesse sur la physiologie de la glande pinéale n'a été observé (Kivela, 1991), quoique cela demeure controversé (Lew, 1987). L'augmentation des taux sanguins maternels de mélatonine observée tout au long de la grossesse (Kivela, 1991, Nakamura *et al.*, 2001) est corrélée avec l'accroissement de la masse du trophoblaste villeux, le principal site de production de mélatonine placentaire comme nous l'avons montré (Lanoix *et al.*, 2008). En outre, la masse du trophoblaste villeux est également accrue chez les grossesses gémellaires et les taux sanguins maternels de mélatonine y sont significativement plus élevés que chez les grossesses avec un seul fœtus (Nakamura *et al.*, 2001). Enfin, après la délivrance du placenta, les taux sanguins maternels de mélatonine chutent rapidement pour rejoindre ceux des femmes non enceintes (Nakamura *et al.*, 2001). Dans l'ensemble, ces études supportent nos résultats et renforcent l'hypothèse originalement émise par Kivela en 1991 mentionnant que le placenta est à l'origine de l'augmentation des concentrations plasmatiques maternelles de mélatonine durant la grossesse (Kivela, 1991). Nous proposons donc, que la production de mélatonine placentaire contribue aux taux retrouvés dans la circulation maternelle et qu'une altération des concentrations maternelles de mélatonine durant la grossesse revête une importance clinique puisqu'ils pourraient indiquer un mauvais fonctionnement placentaire et donc une altération du développement fœtal. L'étude de la mélatonine placentaire présente un intérêt certain dans les complications de la grossesse associée à un défaut du placenta et présentant des taux maternels de mélatonine significativement réduits en comparaison à la grossesse normale, telles que la prééclampsie et la restriction de croissance intra-utérine (Nakamura *et al.*, 2001, Tranquilli *et al.*, 2004).

Dans la même étude, nous avons également démontré l'expression des récepteurs MT1, MT2 et RORα de la mélatonine dans le placenta humain normal à terme (**Chapitre 1**). La localisation des récepteurs MT1 et MT2 a été démontrée dans les cytotrophoblastes villeux, le syncytiotrophoblaste, les capillaires fœtaux et le mésenchyme alors que l'expression du récepteur RORα a été démontrée dans le noyau des cytotrophoblastes villeux, du syncytiotrophoblaste et des capillaires fœtaux. Une autre équipe a identifié l'expression du récepteur MT1 dans le placenta humain à terme, supportant nos résultats (Correa *et al.*, 2009). D'autres auteurs ont démontré l'expression de l'ARNm des récepteurs MT1 et MT2 dans des cellules isolées de placenta humain de premier trimestre qu'ils ont identifiées comme des cellules trophoblastiques (Iwasaki *et al.*, 2005). Par contre, dans cette étude, le type de cellules isolées n'a pas été purifié et leur identité n'a pas été confirmée. On ne peut donc affirmer qu'il s'agit de cellules trophoblastiques. Il serait donc important de caractériser la production de mélatonine et l'expression de ses récepteurs chez les différents types de cellules trophoblastiques du placenta de premier trimestre de la grossesse. Nous avons entrepris cette caractérisation et les résultats préliminaires montrent que l'ARNm et la protéine des récepteurs MT1, MT2 et RORα sont exprimés dans les cytotrophoblastes extravilleux, les cytotrophoblastes villeux et le syncytiotrophoblaste de placentas issus du premier trimestre de la grossesse (**Annexe 1**). Ces résultats indiquent également une localisation différentielle des récepteurs entre les différents types de cellules trophoblastiques ainsi qu'entre les cytotrophoblastes villeux et le syncytiotrophoblaste de premier trimestre de la grossesse et à terme. Cette localisation différentielle suggère un rôle et/ou mode d'action différent des récepteurs de la mélatonine entre les différents types de cellules et selon le terme de la grossesse. Cela demeure toutefois hypothétique. Il serait également intéressant de déterminer la production de mélatonine des cellules trophoblastiques provenant de placentas du premier trimestre de la grossesse. Des études ont également montré l'expression du récepteur MT1 de la mélatonine dans le placenta de rats (Lee *et al.*, 1999, Lee *et al.*, 2003). Le rôle de la mélatonine et de ses récepteurs

placentaires demeure peu connu. Mes travaux de maîtrise montrent que la mélatonine régule la sécrétion de la hCG dans les lignées cellulaires de choriocarcinomes placentaires humains JEG-3 et BeWo, des modèles *in vitro* de cytotrophoblastes extravilleux et villeux respectivement (Lanoix *et al.*, 2006). Bien que le type de cellules trophoblastiques n'ait pas été identifié, l'étude par Iwasaki *et al.* a en outre montré que la mélatonine participe à la régulation des fonctions placentaires en modulant l'expression de la hCG (Iwasaki *et al.*, 2005), supportant mes travaux de maîtrise. Chez le rat, la mélatonine a également été démontrée comme modulatrice de la sécrétion d'hormones, dont des analogues de la prolactine et de la hPL (Lee *et al.*, 1999, Lee *et al.*, 2003). Ces études indiquent un rôle de modulateur endocrinien de la mélatonine dans la grossesse. Par contre, une des principales propriétés de la mélatonine est son importante action cytoprotectrice (Pandi-Perumal *et al.*, 2006). Chez le rat, il a été montré que la mélatonine protège les cellules placentaires contre les dommages induits par de l'ischémie-réperfusion (I/R). En effet, Okatani *et al.* a démontré que la mélatonine renverse les dommages oxydatifs aux mitochondries des placentas de rats induits par l'I/R (Okatani *et al.*, 2001a). De plus, la même équipe a démontré que l'administration de mélatonine aux rates enceintes inhibait significativement le stress oxydatif et l'apoptose mitochondriale ainsi que la restriction de croissance fœtale (Nagai *et al.*, 2008). Chez un modèle d'altération mitochondriale placentaire chez le rat causé par une cholestase maternelle, l'administration de mélatonine renverse la diminution de l'expression des enzymes antioxydantes, la peroxydation des lipides et le niveau de glutathion ainsi que l'apoptose mitochondriale induite par la voie de Bax/Bcl-2 (Perez *et al.*, 2007). Enfin, chez des rates enceintes sous-alimentées, la mélatonine renverse également la diminution de l'expression des enzymes antioxydantes et la restriction de la croissance fœtale (Richter *et al.*, 2009). Chez l'humain, la mélatonine inhibe la peroxydation des lipides dans les mitochondries isolées de placentas humains normaux à terme (Milczarek *et al.*, 2010, Milczarek *et al.*, 2000). Il n'est malheureusement pas possible de savoir de quel type de cellules il s'agit, car des homogénats de tissus placentaires ont été utilisés. De plus, chez des femmes enceintes

qui ont reçu de la mélatonine avant l'accouchement, une augmentation de l'expression de la GPx dans le chorion a été démontrée (Okatani *et al.*, 2001a), renforçant le potentiel cytoprotecteur de la mélatonine dans le placenta. Le rôle cytoprotecteur de la mélatonine dans le placenta humain demeure tout de même peu étudié, en particulier dans le trophoblaste villeux où il n'avait jamais été caractérisé avant la présente étude.

Dans le chapitre 2, les effets cytoprotecteurs de la mélatonine sur l'apoptose mitochondriale du trophoblaste villeux causée par une H/R ont été étudiés (**Chapitre 2**). Ce modèle *in vitro* de perturbation de l'homéostasie du trophoblaste villeux a été choisi, car il reproduit les altérations du trophoblaste présentes dans les complications de la grossesse, notamment la prééclampsie (Hung *et al.*, 2006a, Hung *et al.*, 2001, Hung *et al.*, 2002). Chez les grossesses compliquées par la prééclampsie, le syndrome maternel provient d'un stress inflammatoire systémique en réponse au relargage de facteurs par le syncytiotrophoblaste dans la circulation maternelle (Redman *et al.*, 2009a). L'apoptose du syncytiotrophoblaste est une cause majeure de l'augmentation de la relâche de débris placentaires dans la prééclampsie (Heazell *et al.*, 2008, Leung *et al.*, 2001, Redman *et al.*, 2000). Une altération de l'équilibre pro-oxydant-antioxydant en faveur de la génération de radicaux libres induit l'apoptose mitochondriale du syncytiotrophoblaste (Allaire *et al.*, 2000, Ishihara *et al.*, 2002, Leung *et al.*, 2001). Chez les femmes enceintes, il y a une augmentation du stress oxydatif en comparaison aux femmes non enceintes qui sont contrôlées par le système de défense antioxydant (Hung *et al.*, 2006b). Cette augmentation du stress oxydatif est accrue et non contrôlée par le système de défense antioxydant dans certaines complications de la grossesse tel que la prééclampsie, tant au niveau placentaire que maternel (Hubel, 1999, Hung *et al.*, Little *et al.*, 1999). La fluctuation de la tension en oxygène au contact du syncytiotrophoblaste, due à la mauvaise implantation du placenta dans la prééclampsie, contribue majoritairement à la génération du stress oxydatif (Hung *et al.*, 2006a, Hung *et al.*, 2001). Cette condition d'hypoxie/réoxygénation, grâce à la

génération de radicaux libres, est donc une des sources de l'apoptose mitochondriale du syncytiotrophoblaste participant à la pathogénèse de la prééclampsie (Hung *et al.*, 2006a, Hung *et al.*, 2002).

Les résultats de la présente étude montrent en effet qu'*in vitro*, des cultures primaires de cytotrophoblastes villeux sous H/R présentent les atteintes caractéristiques de la prééclampsie, soit une augmentation des niveaux de radicaux libres conjugués à une diminution des défenses antioxydantes (**Chapitre 2**). Cette hausse du stress oxydatif active des facteurs de transcriptions, tels que NF-κB et p53, qui enclenchent la cascade conduisant à l'apoptose par la voie de la mitochondrie. L'efficacité de l'H/R comme agent induisant un stress dans le tissu placentaire a été démontrée *in vitro* ou elle stimule la production d'espèces réactives oxygénées (Cindrova-Davies, 2009, Cindrova-Davies *et al.*, 2007), inhibe les défenses antioxydantes (He *et al.*, 2009, Matsunami *et al.*, 2009) et accroît l'apoptose par la voie de la mitochondrie (Cindrova-Davies *et al.*, 2007, Hung *et al.*, 2006a, Hung *et al.*, 2002). Nous avons démontré que la mélatonine maintient l'équilibre pro-oxydant-antioxydant, empêche l'activation de NF-κB et p53, qui sont sensibles au stress oxydatif, et inhibe ainsi significativement l'apoptose mitochondriale chez le syncytiotrophoblaste en H/R pour être rétablie à des taux similaires à la normoxie. Dans différents tissus, il a en effet été montré que la mélatonine est un puissant agent cytoprotecteur (Pandi-Perumal *et al.*, 2006). La mélatonine diminue les niveaux de pro-oxydants et maintient les défenses antioxydantes (Bonnefont-Rousselot *et al.*, 2010, Choi *et al.*, 2011, Nagi *et al.*, 2002, Rodriguez *et al.*, 2004, Tomas-Zapico *et al.*, 2005, Turkoz *et al.*, 2004, Zhou *et al.*, 2008). Elle inhibe ainsi l'activation de facteurs de transcriptions sensibles aux stress oxydatifs, tels que NF-κB, p53 et HIF-1 (Chuang *et al.*, 1996, Das *et al.*, 2008, Das *et al.*, 2010, Park *et al.*, 2009) et prévient l'activation de cascades apoptotiques de la voie de la mitochondrie (Radogna *et al.*, 2008). Il serait intéressant de poursuivre la caractérisation de l'effet de la mélatonine sur la cascade apoptotique induite par l'H/R. Les kinases sensibles au stress oxydatif p38 et

SAPK/JNK est une avenue potentielle (Matsuzawa *et al.*, 2005). Elles sont activées par l'H/R dans le syncytiotrophoblaste (Cindrova-Davies, 2009, Cindrova-Davies *et al.*, 2007) et leur activité est augmentée dans les placentas de grossesses compliquées par la prééclampsie, suggérant qu'elles pourraient stimuler l'induction de l'apoptose mitochondriale (Shin *et al.*, 2009).

Le syncytiotrophoblaste est le présumé responsable (Kivela, 1991, Lanoix *et al.*, 2008) de la hausse des taux sanguins maternels de mélatonine durant la grossesse (Kivela, 1991, Nakamura *et al.*, 2001) et ces taux sont significativement réduits dans la prééclampsie (Nakamura *et al.*, 2001, Tranquilli *et al.*, 2004). Il serait donc intéressant de caractériser la production de mélatonine *in vitro* dans le syncytiotrophoblaste soumis à une H/R en comparaison à la normoxie. Dans l'ensemble, les résultats de la présente étude montrent que la mélatonine prévient les altérations de l'homéostasie du syncytiotrophoblaste conduisant à l'apoptose mitochondriale qui sont impliquées dans la pathogénèse de la prééclampsie. Nous avons également publié des travaux dans un article de revue (**Annexe 2.2**) qui supporte ce rôle protecteur de la mélatonine envers l'apoptose mitochondriale du syncytiotrophoblaste (Lanoix *et al.*, 2012b). En effet, la mélatonine renverse significativement l'expression d'éléments de la cascade apoptotique mitochondriale, tels que Bax/Bcl-2, la caspase 9 ainsi que la caspase 3, dans le syncytiotrophoblaste entrant spontanément en apoptose après différenciation. Ces effets de la mélatonine sont dépendants de ses récepteurs MT1 et/ou MT2 car le luzindole, un antagoniste sélectif des récepteurs MT1/MT2, renverse significativement l'effet de la mélatonine. Ces résultats montrent qu'en absence d'agent stresseur, le rôle protecteur de la mélatonine est dépendant de l'activation de ses récepteurs. *In vitro*, sans la fusion continue des cytotrophoblastes villeux, la durée de vie du syncytiotrophoblaste n'est que de quelques jours (Castellucci *et al.*, 1990, Crocker *et al.*, 2004). En absence d'agent stresseur, la mélatonine ne rend toutefois pas le syncytiotrophoblaste immortel, elle ne fait qu'améliorer sa survie. Ces

résultats suggèrent qu'en présence d'un agent stresseur, la mélatonine pourrait promouvoir la survie du syncytiotrophoblaste, supportant les résultats obtenus avec le modèle d'H/R (**Chapitre 2**).

Dans la présente étude, la mélatonine placentaire (production et expression de ses récepteurs) a également été caractérisée dans les placentas de grossesses compliquées par la prééclampsie en comparaison à la grossesse normotensive (**Chapitre 3**). Dans un premier temps, les techniques de RT-qPCR et d'immunobuvardage de type Western semi-quantitatif pour comparer l'expression de l'ARNm ou de la protéine entre une condition normale et pathologique ont été validées. En effet, l'utilisation de ces deux techniques repose sur la normalisation de l'expression du gène ou de la protéine cible sur des gènes ou des protéines de références. L'expression des gènes et des protéines de références utilisées ne doit pas varier entre la condition normale et pathologique pour obtenir des résultats valides (Cleal *et al.*, 2009, Meller *et al.*, 2005, Murthi *et al.*, 2008). Nos résultats démontrent que l'utilisation du colorant général à protéine amido black est le marqueur protéique dont l'expression est la plus stable entre les placentas de grossesses normotensives et compliquées par la prééclampsie (**Chapitre 3.1**). De plus, il est essentiel de suivre rigoureusement les lignes directrices du *Minimum Information for Publication of Quantitative Real-Time PCR Experiment* (MIQE) afin d'obtenir des résultats d'expression génique valable et précis (Bustin *et al.*, 2009). Pour ce faire, la moyenne géométrique de l'expression d'au moins trois gènes de référence doit être utilisée pour quantifier précisément l'expression génique entre des placentas de grossesses normotensive et compliquées par la prééclampsie (**Chapitre 3.2**). En plus de la stabilité des gènes de références, un autre paramètre essentiel à valider pour déterminer l'expression de gènes par RT-qPCR est l'intégrité des échantillons d'ARN qui seront analysés (Bustin *et al.*, 2009, Vermeulen *et al.*, 2011). L'utilisation d'environ 25% d'échantillons d'ARN montrant des signes de dégradation abolit complètement une différence d'expression significative entre les placentas de

grossesses normotensives et compliquées par la prééclampsie (**Chapitre 3.2**). Ces deux études démontrent qu'il est important de vérifier la stabilité des gènes ou des protéines de références pour obtenir des résultats valides et reproductibles et que l'omission de le faire peut conduire à l'obtention de résultats différents et même significativement opposés à la réalité. Conséquemment, les résultats d'expression de gènes ou de protéines que nous avons obtenus en caractérisant la production de mélatonine et l'expression de ses récepteurs entre les placentas de grossesses normotensives et compliquées par la prééclampsie sont précis (**Chapitre 3**).

Nos résultats démontrent une diminution significative de la production placentaire de mélatonine dans les placentas de grossesses compliquées par la prééclampsie en comparaison avec la grossesse normotensive (**Chapitre 3**). Cette altération de la production placentaire de mélatonine est due à un défaut d'expression et d'activité de AANAT, enzyme limitante de la synthèse de la mélatonine. La diminution de la production placentaire de mélatonine dans la grossesse compliquée par la prééclampsie en comparaison à la grossesse normotensive est corrélée à la diminution des taux sanguins maternels de mélatonine observés dans cette complication de la grossesse (Nakamura *et al.*, 2001, Tranquilli *et al.*, 2004). Ces résultats appuient de nouveau l'hypothèse que le placenta est responsable de l'augmentation des niveaux de mélatonine dans le sang maternel durant la grossesse (Kivela, 1991). Nous avons également démontré une diminution significative de l'expression de l'ARNm et de la protéine des récepteurs MT1 et MT2 dans les placentas de grossesses compliquées par la prééclampsie. Cette dérégulation de l'expression de MT1 et MT2 dans les placentas de prééclampsie pourrait être attribuable à la diminution des taux de mélatonine qui y sont observés (Yerer *et al.*, 2010). La mélatonine induit une partie de son importante action cytoprotectrice par l'activation de ses récepteurs MT1 et MT2 (Pandi-Perumal *et al.*, 2006). Il a notamment été démontré que la mélatonine protège le myocarde contre les dommages causés par une ischémie/réperfusion par

des mécanismes à la fois dépendants et indépendants de ses récepteurs (Sallinen *et al.*, 2007). De plus, la capacité de la mélatonine à promouvoir la survie cellulaire chez plusieurs tissus est induite à la fois dépendamment et indépendamment de ses récepteurs (Hardeland *et al.*, 2009, Rodriguez *et al.*, 2004, Tan *et al.*, 1999, Tan *et al.*, 1998, Tomas-Zapico *et al.*, 2005). Conséquemment, dans les placentas de grossesses compliquées par la prééclampsie, la diminution significative de production de mélatonine, conjuguée à la diminution significative de l'expression de ses récepteurs MT1 et MT2, indique la perte de ses propriétés cytoprotectrices. Dans l'ensemble, nos travaux et la littérature actuelle, montrant que la mélatonine protège le placenta contre un stress oxydatif (Lee *et al.*, 2003, Milczarek *et al.*, 2010, Milczarek *et al.*, 2000), convergent vers une implication de l'altération de la production de mélatonine et de sa signalisation dans les processus pathologiques conduisant au syndrome maternel de la prééclampsie. La diminution de la production de mélatonine et de l'expression de ses récepteurs dans les placentas de prééclampsie indique que la mélatonine pourrait être un facteur aggravant dans la pathogénèse de cette complication obstétricale. Pour confirmer ces résultats, la production de mélatonine et l'expression de ses récepteurs MT1 et MT2 devront être analysées dans des cultures primaires de trophoblastes villeux provenant de grossesses compliquées par la prééclampsie en comparaison à la grossesse normotensive. En perspective, il serait également intéressant d'étudier l'effet de la mélatonine sur l'apoptose mitochondriale du syncytiotrophoblaste dans des cultures primaires de trophoblastes villeux provenant de grossesses compliquées par la prééclampsie en comparaison à la grossesse normotensive.

Nous proposons que la mélatonine puisse être un outil diagnostic ou un biomarqueur pour le dépistage précoce de la prééclampsie. Il n'existe actuellement aucun traitement définitif pour la prééclampsie, sauf la délivrance du placenta (American College of Obstetricians and Gynecologists, 2002, National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy, 2000). Plusieurs auteurs ont proposé l'utilisation de la mélatonine

pour traiter la prééclampsie (Milczarek *et al.*, 2010, Okatani *et al.*, 2001a, Wakatsuki *et al.*, 2001). Des antioxydants, dont les vitamines C et E, ont été utilisés pour prévenir les dommages induits par l'H/R chez la prééclampsie. Par contre, malgré leur efficacité *in vitro* pour contrer les altérations du trophoblaste villeux induites par de l'H/R (Cindrova-Davies, 2009, Cindrova-Davies *et al.*, 2007), *in vivo*, les vitamines n'ont montré aucun bénéfice dans la prévention de la prééclampsie (Bortolotti *et al.*, 1990, Matsunami *et al.*, 2009, Poston *et al.*, 2006, Reiter *et al.*, 2009, Rumbold *et al.*, 2006, Tuuli *et al.*, 2011). Deux explications retenues pour expliquer l'inefficacité des vitamines pour prévenir ou améliorer la prééclampsie sont un dosage inadéquat et leur accessibilité (Reiter *et al.*, 2009). Contrairement aux vitamines, la mélatonine est un puissant antioxydant *in vivo* à faibles doses (Bubenik *et al.*, 1998). Des études ont montré que des doses 200 fois plus élevées de vitamines E que de mélatonine sont requises pour avoir les mêmes effets cytoprotecteurs (Reiter *et al.*, 2009, Tamura *et al.*, 2008). De plus, la mélatonine est une hormone amphiphile qui peut pénétrer dans chaque parties de la cellule et traverser toutes les barrières morphophysiologiques, dont la barrière placentaire (Okatani *et al.*, 1998, Pardridge *et al.*, 1980), contrairement à la vitamine E (Bortolotti *et al.*, 1990). Une étude montre en outre que la mélatonine améliore l'action antioxydante de la vitamine C et E chez des mitochondries isolées de placentas humains normaux à terme et que l'utilisation combinée des trois molécules possède la plus forte action antioxydante (Milczarek *et al.*, 2010). La mélatonine présente donc une avenue potentielle pour prévenir ou traiter la prééclampsie ainsi que d'autres complications de la grossesse caractérisées par une altération de l'homéostasie du trophoblaste villeux, tel que la restriction de croissance intra-utérine ou le diabète gestationnel mellitus (Figueroa-Quevedo *et al.*, 2011, Heazell *et al.*, 2011).

En conclusion, la présente étude a démontré la production *de novo* de la mélatonine ainsi que l'expression de ses récepteurs MT1 et MT2 par le trophoblaste villeux du placenta humain à terme. La présence du système mélatonine dans le placenta humain suggère un rôle

auto/intra/para/endocrine de la mélatonine produite localement. Le mode d'action de la mélatonine placentaire sur la prolifération, l'invasion et la différenciation des différents types de cellules trophoblastiques ainsi que sur le développement fœtal reste à être étudié. Les présents travaux suggèrent que la mélatonine plasmatique maternelle est majoritairement d'origine placentaire. La présente étude a également permis de montrer une action protectrice de la mélatonine contre l'apoptose mitochondriale par la voie de Bax/Bcl-2 induite par de l'H/R dans le syncytiotrophoblaste en culture primaire. Les autres voies de l'apoptose mitochondriale de même que les différentes voies de l'apoptose induite par les récepteurs de morts (apoptose extrinsèque) demeurent à être étudiées. Il serait également intéressant d'utiliser une approche antisens à l'aide de petits ARN interférents (siRNA) afin de déterminer l'implication des récepteurs MT1 et MT2 dans l'activation de ces voies de signalisation. Enfin, les présents travaux ont démontré une réduction significative de la production de mélatonine de même que de l'expression de ses récepteurs dans les placentas de grossesses compliquées par la prééclampsie en comparaison à la grossesse normotensive, suggérant un rôle de la mélatonine dans la pathogénèse de la prééclampsie. La réduction des taux plasmatiques maternels de mélatonine pourrait donc être un outil diagnostic précoce pour identifier les grossesses compliquées par la prééclampsie. La mélatonine pourrait ainsi être un traitement potentiel de la prééclampsie chez les femmes enceintes où des taux plasmatiques maternels de mélatonine auront été identifiés.

Dans l'ensemble, la présente étude a permis de mieux cerner le rôle de la mélatonine dans le placenta humain en situation normale et dans la grossesse compliquée par la prééclampsie, et d'ouvrir la voie à un nouveau biomarqueur potentiel de la prééclampsie et d'établir la mélatonine comme une nouvelle piste pour prévenir ou traiter la prééclampsie.

CONTRIBUTION À L'AVANCEMENT DES CONNAISSANCES

Les résultats obtenus au cours de ce projet de doctorat contribuent à l'avancement des connaissances de plusieurs façons. Dans un premier temps, ils ont permis de démontrer que le placenta humain produit *de novo* la mélatonine et que la majorité de l'importante production placentaire de mélatonine a lieu chez le trophoblaste villeux, en particulier dans le syncytiotrophoblaste. Des études supplémentaires sont requises pour caractériser les mécanismes de régulation de la production de mélatonine chez le placenta ainsi que pour déterminer la production de mélatonine dans le placenta de premier trimestre de la grossesse. De plus, ces travaux ont démontré l'expression des récepteurs MT1, MT2 et RORα de la mélatonine dans les placentas normaux de premier trimestre de la grossesse et à terme; tant chez les cytotrophoblastes extravilleux, les cytotrophoblastes villeux et le syncytiotrophoblaste. Les niveaux d'expression différentiels de ces récepteurs chez les différents types de cellules trophoblastiques ainsi que selon le terme de la grossesse semblent indiquer qu'ils auraient des rôles différents selon l'endroit et le moment où ils sont exprimés. Des études supplémentaires devront être effectuées afin de caractériser le rôle de la mélatonine et de ses récepteurs au cours de la grossesse. Nous avons démontré que la mélatonine prévient l'apoptose mitochondriale du syncytiotrophoblaste stimulée par un état d'hypoxie/réoxygénation, un phénomène impliqué dans la pathogénèse de la prééclampsie. Des études plus approfondies sont nécessaires afin de poursuivre le décryptage des mécanismes par lesquels la mélatonine induit ces effets cytoprotecteurs. Ces résultats mettent en évidence un rôle bénéfique de la mélatonine placentaire dans le bon déroulement de la grossesse et de la croissance fœtale et indiquent que la production ou la signalisation de la mélatonine pourrait être altérée chez les placentas de grossesses compliquées par la prééclampsie. Nos travaux ont en effet permis de montrer une diminution significative de la production de mélatonine ainsi que de l'expression de ses récepteurs chez les placentas de grossesses compliquées par la prééclampsie en

comparaison avec la grossesse normotensive. Ces altérations indiquent une perte du rôle cytoprotecteur de la mélatonine envers le syncytiotrophoblaste chez la prééclampsie et convergent vers une implication de la mélatonine dans la pathogénèse de la prééclampsie. Des études additionnelles pourront caractériser les fonctions cytoprotectrices de la mélatonine chez le syncytiotrophoblaste de grossesse compliqué par la prééclampsie. Enfin, nous proposons qu'une diminution des taux de mélatonine dans la circulation maternelle puisse être envisagée comme biomarqueur précoce permettant de révéler des atteintes à la survie du syncytiotrophoblaste qui conduisent au syndrome maternel de la prééclampsie. Dans l'ensemble, ces travaux représentent un avancement majeur dans la compréhension du rôle de la mélatonine placentaire dans le déroulement de la grossesse et de la croissance fœtale et supportent l'utilisation de la mélatonine comme nouvelle stratégie thérapeutique pour prévenir ou améliorer la prééclampsie; la principale cause de mortalité et morbidité maternelle et périnatale.

ANNEXE 1 – RÉSULTATS PRÉLIMINAIRE:

MELATONIN RECEPTORS AND SYNTHESIZING ENZYMES EXPRESSION IN HUMAN PLACENTAL TISSUE AND TROPHOBLAST CELLS DURING PREGNANCY

Dave Lanoix, Mélanie Cocquebert, Thierry Fournier et Cathy Vaillancourt

Résultats préliminaires destinés à être un manuscrit pour soumission dans J Pineal Res.

MELATONIN RECEPTORS AND THE TROPHOBLAST: A DIFFERENTIAL RELATIONSHIP

Lanoix D, Cocquebert M, Fournier T, Vaillancourt C

METHODS

Placenta tissues

Placental tissues from patients who voluntarily and legally chose to terminate pregnancy during the first trimester (8–12 week of gestation) were obtained from Broussais Hospital, Paris, France. Third trimester (39-41 week of gestation) placentas were obtained immediately after spontaneous vaginal deliveries from uncomplicated pregnancies at the CHUM-St-Luc Hospital, Montreal, QC, Canada. All placentas were obtained with informed patient consent and approval of ethical committees.

Isolation and purification of first trimester villous and extravillous cytotrophoblasts

evCTB and vCTB were isolated from the same chorionic villi as previously described [1].

Isolation and purification of third trimester villous cytotrophoblasts

VCT were isolated and purify as previously described [2].

Localization of melatonin receptors in placental tissue

The expression melatonin receptors in placental tissue were determined by immunohistochemistry as described previously [1].

Expression of melatonin receptors between the types of trophoblast cells

The differential expression of melatonin receptors in primary trophoblast cells was determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) as previously described (Lanoix et al. 2012 Mol Biotechnicol). Total RNA was extracted from frozen placental tissues

using Aurum Total RNA mini kit (Bio-Rad). RNA integrity was assessed with the Experion Automated Electrophoresis Station (Bio-Rad). RNA samples that successfully met the quality control standards were reverse transcribed to cDNA with the iScript cDNA synthesis kit according to manufacturer's instructions (Bio-Rad). Primers were designed using Oligo 6 software (Molecular Biology Insights, Cascade, USA) and their specificity was determined with Primer-Blast program (http://www.ncbi.nlm.nih.gov/tools/primerblast/). Amplicons were tested for potential secondary structure using mfold web server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form). Primers specificity was confirmed by agarose gel electrophoresis after RT-qPCR reaction. Validated sequences are shown in **Table 2.** SsoFast EvaGreen Supermix (Bio-Rad) was used for amplification. Reactions were run on a CFX96 Real-Time PCR Detection System (Bio-Rad). Stability of reference genes was analysed using geNorm software [3].

Intracellular localization of melatonin receptors in the trophoblast cells

Trophoblast cells were immunolabeled for melatonin receptor as previously described with minor modifications (Lanoix et al 2006). Briefly, cells were fixed for 20 min in 4% PBS-buffered paraformaldehyde. Cells were rinsed with PBS and blocked for 1h at RT in blocking buffer (0.5% donkey serum). Then, cells were stained with primary antibodies diluted in blocking buffer overnight at 4°C as described in **Table 1.** Alexa-Fluor 488-conjugated secondary antibodies (Invitrogen) were incubated for 1h at RT (**Table 1**). Nucleuses were counterstained using DRAQ5 according to manufacturer's instruction (Biostatus).

RESULTS

Localization of melatonin receptors in first trimester and term placental tissue

Melatonin receptor expression in first trimester and term placental tissue was assessed by immunohistochemistry. **Figure 1** and **2** shows that MT1, MT2 and RORα melatonin receptors were expressed in first trimester and term placental tissue. In first trimester placentas, melatonin receptors are strongly expressed in villous cytotrophoblast compared to syncytiotrophoblast (**Figure 1**). However, in term placentas, the opposite is observed (**Figure 2**). Melatonin receptors are strongly expressed in syncytiotrophoblast compared to villous cytotrophoblast. MT1, MT2 and RORα melatonin receptors are expressed in first trimester extravillous cytotrophoblasts (**Figure 1**). The localization of the extravillous cytotrophoblast and syncytiotrophoblast was confirmed using cytokeratin-7 and human chorionic gonadotrophin antibodies respectively (**Figure 1, 2**) [4]. In term placentas, MT1, MT2 and RORα melatonin receptors are also expressed in fetal capillaries (**Figure 2**). The localization of fetal capillaries was confirmed by the expression of CD34 [4].

Expression of melatonin receptors in trophoblast cells

Primary extravillous and villous trophoblast cells were isolated from first trimester and term placentas. MT1, MT2 and RORα melatonin receptors expression in primary trophoblast cells was confirmed using quantitative RT-PCR (**Figure 3, 4**). The geNorm software was use to determine the most stably expressed genes. Melatonin receptor expression was then normalized against HPRT1, PPIA and TBP. In first trimester trophoblast cells, melatonin receptors expression is significantly higher in villous trophoblast than in the syncytiotrophoblast and the extravillous

cytotrophoblast (**Figure 3**). Conversely, in term trophoblast cells, melatonin receptor expression is significantly higher in the syncytiotrophoblast than in villous cytotrophoblast (**Figure 4**).

Intracellular localization of melatonin receptors in trophoblast cells

Intracellular localization of melatonin receptor was determined using immunofluorescence analysis in first trimester and term primary trophoblast cells (**Figure 5, 6**). In first trimester extravillous cytotrophoblast, MT1 is localized in the cytoplasm membrane and the cytoplasm while MT2 is localized in the cytoplasm membrane, the cytoplasm and the nucleus (**Figure 5, 6**). In first trimester placenta, MT1 and MT2 melatonin receptors were strongly localized in villous trophoblast cells compared to the syncytiotrophoblast (**Figure 5**). In opposition, in term placentas, MT1 and MT2 melatonin receptors are strongly localized in the syncytiotrophoblast than in the villous trophoblast (**Figure 6**). RORα melatonin receptors were localized in the cytoplasm and nucleus in both first trimester and term trophoblast cells (**Figure 5, 6**).

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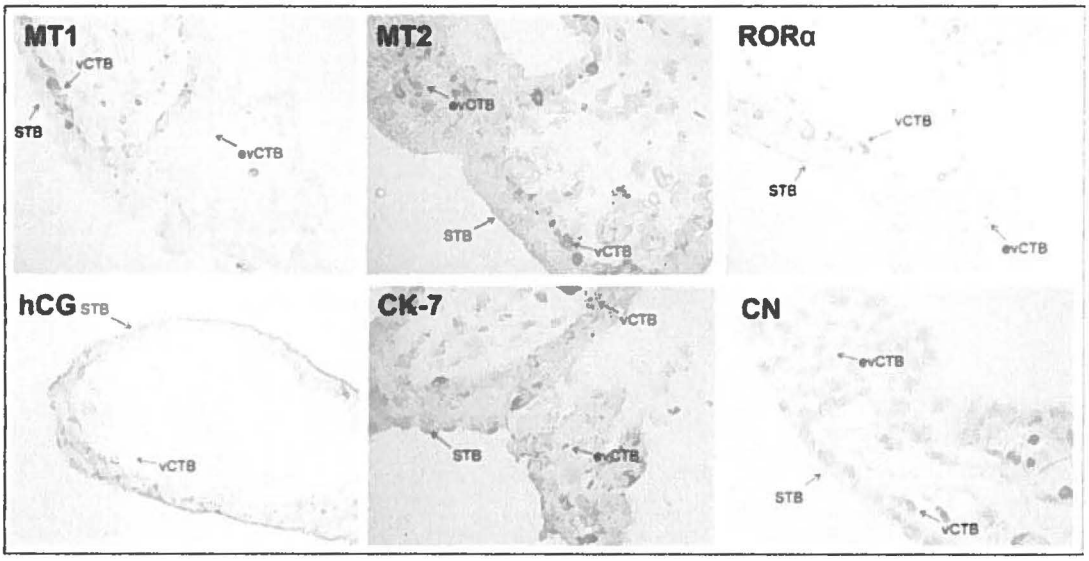
MT1
vCTB
STB
evCTB
MT2
evCTB
STB
vCTB
RORα
vCTB
STB
evCTB
hCG
STB
vCTB
CK-7
vCTB
STB
evCTB
CN
evCTB
STB
vCTB

Figure 1.

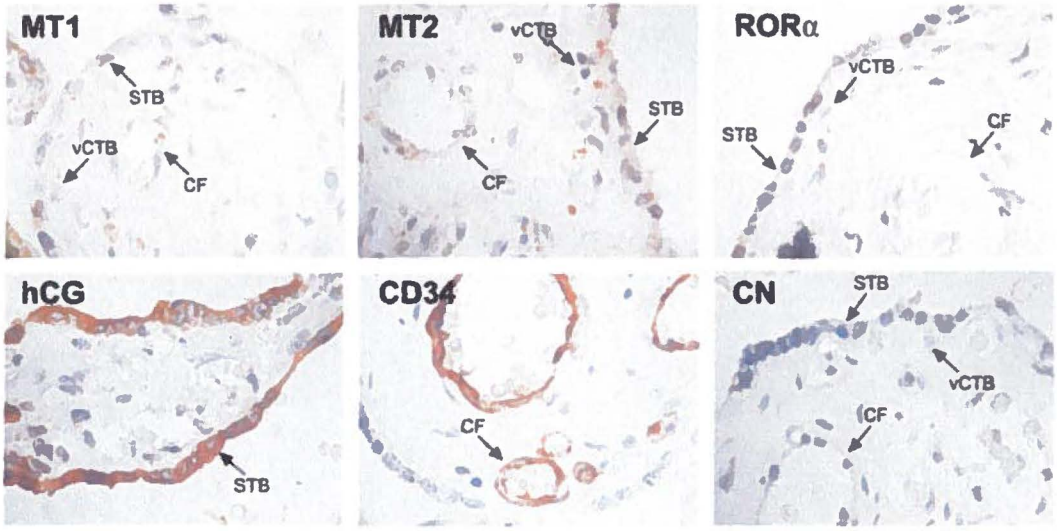
MT1
STB
vCTB
CF
MT2
vCTB
STB
CF
RORα
vCTB
STB
CF
hCG
STB
CD34
CF
CN
STB
vCTB
CF

Figure 2.

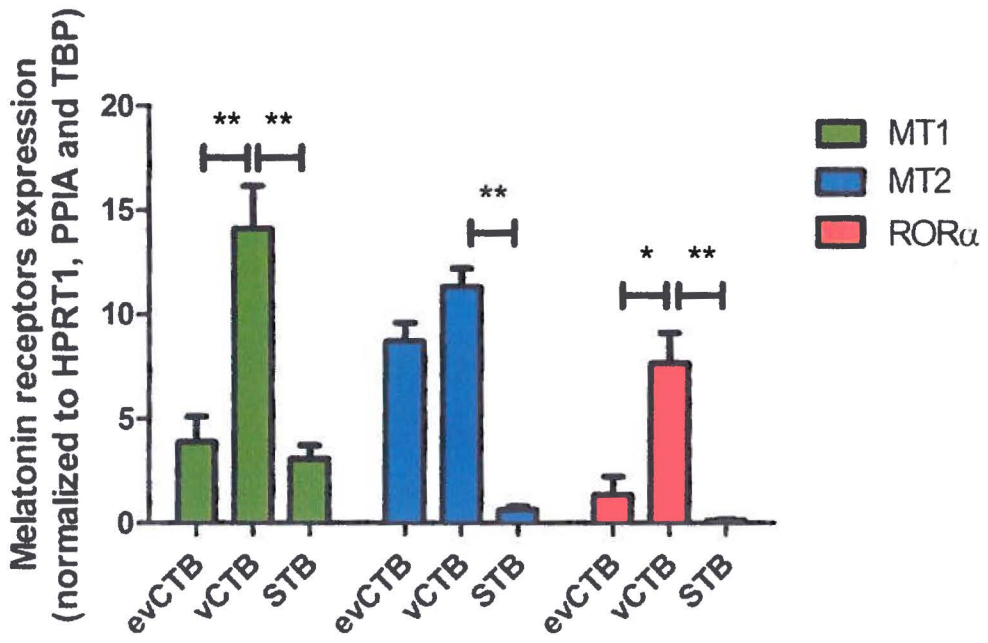
Melatonin receptors expression
(normalized to HPRT1, PPIA and TBP)
20
15
10
5
0
** **
**
* **
MT1
MT2
RORα
evCTB
vCTB
STB
evCTB
vCTB
STB
evCTB
vCTB
STB

Figure 3.

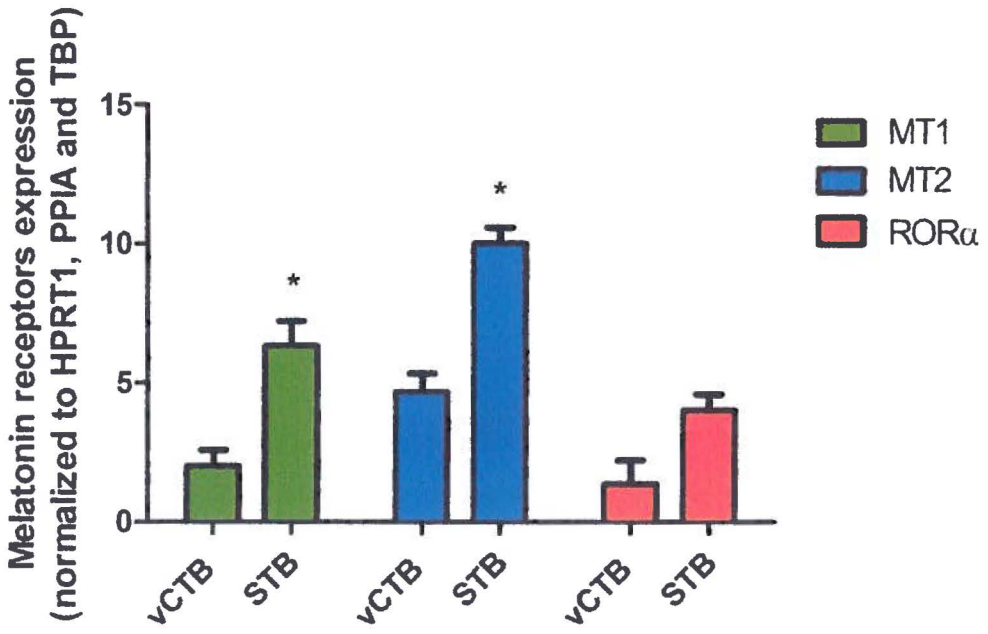
Melatonin receptors expression
(normalized to HPRT1, PPIA and TBP)
15
10
5
0
*
*
MT1
MT2
RORα
vCTB
STB
vCTB
STB
vCTB
STB

Figure 4.

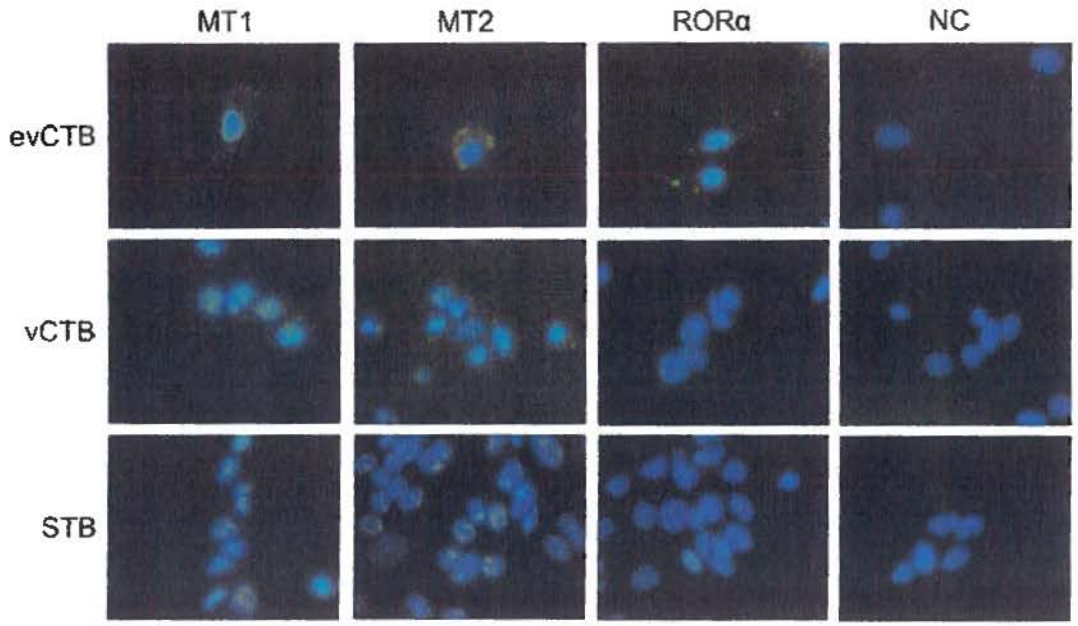

Figure 5.

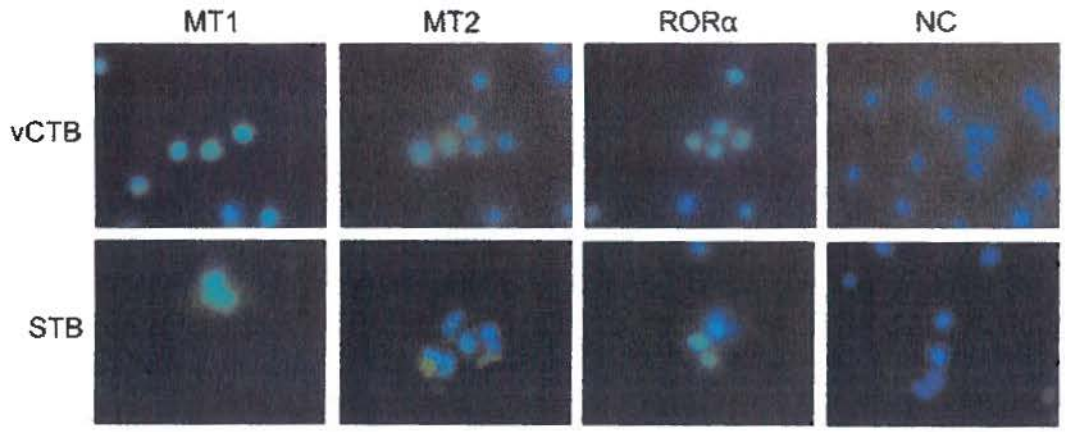

Figure 6.

ANNEXE 2 - LETTRE À L'ÉDITEUR:
CELL CULTURE MEDIA FORMULATION AND SUPPLEMENTATION AFFECT VILLOUS TROPHOBLAST HCG RELEASE

Dave Lanoix et Cathy Vaillancourt

Placenta. 2010, 31 :558-559

Lanoix D, Vaillancourt C. Cell culture media formulation and supplementation affect villous trophoblast HCG release.
Placenta. 2010 Jun;31(6):558-9.
doi:10.1016/j.placenta.2010.04.004. Epub 2010 May 10. PubMed PMID: 20452669

CELL CULTURE MEDIA FORMULATION AND SUPPLEMENTATION AFFECT VILLOUS TROPHOBLAST hCG RELEASE

Résumé de l'article en français

Cette lettre à l'éditeur ne comporte pas de résumé.

Contribution de l'étudiant

L'étudiant a réalisé toutes les expériences présentées dans cet article, a analysé les résultats, rédigé l'article, a participé au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

ANNEXE 3- ARTICLE DE REVUE: MELATONIN: THE SMART KILLER- THE HUMAN TROPHOBLAST AS AMODEL

Dave Lanoix, Andrée-Anne Lacasse, Russel J. Reiter et Cathy Vaillancourt

Molecular and Cellular Endocrinology. 2012, 348:1-11

Lanoix D, Lacasse AA, Reiter RJ, Vaillancourt C. Melatonin: the smart killer:
the human trophoblast as a model.
Mol Cell Endocrinol. 2012 Jan 2;348(1):1-11.
doi: 10.1016/j.mce.2011.08.025. Epub 2011 Aug 26. Review. PubMed PMID: 21889572.

MELATONIN: THE SMART KILLER- THE HUMAN TROPHOBLAST AS AMODEL

Résumé de l'article en français

La mélatonine peut à la fois induire l'apoptose intrinsèque dans les cellules tumorales alors qu'elle l'inhibe dans les cellules non tumorales. La mélatonine tue les cellules tumorales en induisant la production d'espèces réactives oxygénées et en activant les voies pro-apoptotiques. En revanche, la mélatonine permet la survie des cellules non tumorales grâce à ses propriétés antioxydantes et à l'inhibition des voies pro-apoptotiques. Dans les cultures primaires de trophoblaste villeux humain, connu comme étant un tissu pseudo tumorale, la mélatonine favorise la survie via l'inhibition de la voie de Bax/Bcl-2 cependant dans la ligné cellulaire de choriocarcinome humain BeWo la mélatonine induit la perméabilisation de la membrane mitochondriale entraînant la mort cellulaire. Ces résultats suggèrent que le trophoblaste est un bon modèle pour étudier les différents effets de la mélatonine sur les voies intrinsèques de l'apoptose. Cette revue décrit les différents effets de la mélatonine sur les voies intrinsèques de l'apoptose dans les cellules tumorales et non tumorales et présente le trophoblaste comme un nouveau système modèle pour l'étude de ces effets de la mélatonine.

Contribution de l'étudiant

L'étudiant a rédigé environ 60% de l'article, conçu et dessiné toutes les figures, participé au choix du journal de publication et aux corrections nécessaires à la publication de la version finale de l'article.

ANNEXE 4 – ARTICLE DE REVUE: PLACENTAL DISORDER IN PREECLAMPSIA: MATERNAL AND PERINATAL OUTCOMES

Dave Lanoix, Sophie Haché, Evemie Dubé, Julie Lafond et Cathy Vaillancourt

in: Pregnancy disorder and perinatal outcomes. 2012, chapter 7. Bentham Science Publishers eBook. Editors: Julie Lafond and Cathy Vaillancourt.

PLACENTAL DISORDER IN PREECLAMPSIA: MATERNAL AND PERINATAL OUTCOMES

Résumé de l'article en français

La prééclampsie, une complication de la grossesse, est une cause majeure de morbidité et de mortalité maternelle et infantile qui affecte environ 3-15% des grossesses dans le monde. Elle est caractérisée par une pression artérielle élevée et la présence de protéine dans l'urine. Cette maladie est d'origine placentaire causant divers problèmes chez la mère et le fœtus. Dans le pire des cas, elle peut même menacer la survie maternelle et fœtale. La prééclampsie est définie comme un syndrome (un ensemble de caractéristiques clinique) et a probablement une origine et une présentation hétérogène. À ce jour, le seul remède connu pour soigner la prééclampsie est l'accouchement accompagné par la délivrance du placenta. L'étiologie complète de la prééclampsie n'est pas connue, la recherche est donc cruciale afin d'en connaître plus sur cette physiopathologie pour permettre le développement de différents traitements, un diagnostic précoce ainsi qu'une approche préventive. Ce chapitre se concentre sur les connaissances actuelles et les découvertes récentes concernant la prééclampsie, particulièrement sur le rôle du placenta dans sa physiopathologie. Ce chapitre présente également les connaissances actuelles sur le diagnostic de la prééclampsie, l'épidémiologie, les facteurs de risques et la pathogénèse avec une emphase particulière sur les conséquences maternelles et périnatales.

Contribution de l'étudiant

L'étudiant a rédigé environ 60% de l'article, conçu et dessiné toutes les figures et participé aux corrections nécessaires et à la publication de la version finale de l'article.

CHAPTER 7

Placental Disorders in Preeclampsia: Maternal and Perinatal Outcomes

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Abstract: Preeclampsia, a disorder of pregnancy, is a leading cause of maternal and infant illness and death affecting about 3-15 % of all pregnancies worldwide. It is characterized by high blood pressure and the presence of protein in the urine. It originates in the placenta and causes variable maternal and fetal problems. At its worst, it may threaten maternal and perinatal survival. Preeclampsia is defined as a syndrome (a pattern of clinical features) and is probably heterogeneous in its origin as it is in its presentation. To date, the only complete cure known for preeclampsia is delivery, accompanied by the removal of the placenta. As the complete etiology of preeclampsia is still unknown, researches are crucial in order to know more about this pathophysiology and to develop different treatments and prediction approaches. This chapter focuses on current knowledge and recent discoveries on preeclampsia, especially on the role of placenta in its physiopathology. The chapter also presents current knowledge concerning preeclampsia diagnosis, epidemiology, risk factors and pathogenesis with an emphasis on maternal and perinatal outcomes related to this most common cause of death for both children and mothers during pregnancy.

Keywords: Preeclampsia, placenta, fetal development, maternal hypertension, proteinuria, trophoblast, intra-uterine growth restriction (IUGR).

INTRODUCTION: EPIDEMIOLOGY AND RISK FACTORS

Preeclampsia is one of the most common pregnancy complication with a worldwide incidence of 3-15% and causes approximately 63 000 deaths annually, mainly in less developed countries [1, 2]. This disorder is the most common cause of death for both children and mothers during pregnancy and is responsible for 15% of premature births in industrialized countries. High blood pressure is relatively common in pregnancy, affecting 12-18% of all pregnancies [3]. About 50% of women with gestational hypertension will develop preeclampsia [4]. Healthy nulliparous women correspond to nearly 75% of cases where the disease is mild and the risk of pregnancy outcome is negligible [5-7]. However, the burden and frequency of preeclampsia are significantly increased with by medical conditions, such as multiparous pregnancy, chronic hypertension, pre-existing diabetes mellitus, pre-gestational thrombophilia and previous preeclampsia. Table 1 summarizes the most commons risk factors associated with increased risk of preeclampsia.

Preeclampsia is often described as a disease of first pregnancies. Indeed, risk of preeclampsia is most important in first pregnancies [34, 35] while it is decreased by pre-conception maternal sperm exposure [36, 37]. Furthermore, conception through assisted reproductive technologies using donor sperm increases the risk of preeclampsia [38, 39]. These studies suggest a major immunological and paternal factor in the pathogenesis of preeclampsia. Moreover, women who have preeclampsia during their first pregnancy have more chances of developing preeclampsia again in the next pregnancy. In fact, when preeclampsia is developed before 30 weeks of gestation during the first pregnancy, the recurrence rate may be as high as 40% in future pregnancies [3].

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Table 1: Risk factors

Maternal risk factors
Multiparous pregnancy [8-10]
Chronic hypertension [11, 12]
Bilateral notches [13]
Pre-existing diabetes mellitus [14, 15]
Previous preeclampsia [16-18]
Pre-gestational thrombophilia [19, 20]
Elevated or low maternal age [21, 22]
Obesity and insulin resistance [23-25]
Familiar history of preeclampsia [26, 27]
Maternal infections [28-30]
Maternal susceptibility genes [31-33]
Paternal risk factors
Primipaternity [34, 35]
Limited sperm exposure [36, 37]
Conception after assisted reproductive technologies [38-40]

CLINICAL SYMPTOMS

Preeclampsia is an heterogeneous and systemic disease, which usually develops after 20 weeks of gestation and prior 48 h postpartum [41]. Clinical guidelines support the distinction into mild and severe preeclampsia, as well as early (before 34 weeks) and late (after 34 weeks) onset of preeclampsia [42, 43]. Typical symptoms of mild preeclampsia include systolic blood pressure (SBP) ≥ 140 mm Hg or diastolic blood pressure (DBP) ≥ 90 mm Hg and proteinuria (≥ 300 mg in 24 h urine sample) [44, 45]. Generally the late onset preeclampsia shows mild symptoms and accounts for more than 80% of all cases worldwide [42]. Severe preeclampsia, which usually includes the early onset type, is described as SBP ≥ 160 mm Hg or DBP ≥ 110 mm Hg on two occasions at least 6 h apart in a woman on bed rest and proteinuria (≥ 5 g in 24 h urine sample) on two random urine samples collected at least 4 h apart [42, 45].

Blood Pressure

In normotensive pregnancies, several changes in uterine blood flow are observed including a decrease in blood pressure and peripheral vascular resistance [46], an increase of maternal blood volume, cardiac output [47] and artery volume flow [48]. These changes are essential to meet metabolic demands from the placenta and to increase the flow of nutrients to the growing fetus [49]. However, preeclamptic pregnant women show impaired endothelium-dependent vasorelaxation, widespread vasoconstriction, high vascular resistance, low cardiac output [50, 51] and a decrease in utero-placental blood flow by up to 50% [52].

Hemodynamic and vascular adaptations are also altered in preeclamptic pregnancies. The parathyroid hormone related protein (PTHrP), which is one of the factors involved in this regulation, shows decreased circulating levels in preeclamptic women [53]. PTHrP plays many roles during the pregnancy, such as the relaxation of uterine arteries [54] and placental calcium transfer [55-57]. In addition, disturbances of the utero-placental renin-angiotensin system (RAS) in pregnancies complicated by preeclampsia could lead to dysfunctional bleeding and reduced utero-placental blood flow [58]. Indeed the circulating RAS plays a key role in regulating blood pressure and electrolyte balance. Furthermore, the utero-placental RAS is important for the regeneration of the endometrium after shedding, and for decidualization, implantation and placentation.

Calcium supplementation reduces blood pressure in pregnant women at risk for hypertensive disorders or with low dietary calcium intake [59-62]. Urinary calcium excretion also correlates to increase blood pressure in preeclampsia [63]. In fact, during normotensive pregnancies, the extracellular fluid volume

expands. In consequence, there is a dilution of calcium and an increased glomerular filtration which causes calcium losses [63].

Proteinuria

Proteinuria is a major dysfunction of preeclampsia, defined as a urinary total protein of ≥ 300 mg in 24 h. Proteinuria is generally associated with glomerular endotheliosis, described as the swelling of the glomerular capillary endothelium that causes decreased glomerular perfusion and filtration rate [41, 64]. It is not permanent and recovers after delivery [65].

Proteinuria is not universally considered obligatory for the diagnosis of preeclampsia [66]. For example, the Australasian Society for the Study of Hypertension in Pregnancy (ASSHP) and the Society of Obstetric Medicine of Australia and New Zealand (SOMANZ) do not required proteinuria [67]. In this case, to be diagnosed for preeclampsia, women need to have hypertension and one of the following clinical features: renal insufficiency, pulmonary edema, liver disease, neurological problems, hematological disturbance, intra-uterine growth restriction (IUGR) or proteinuria. On the other hand, the International Society for the Study of Hypertension in Pregnancy (ISSHP) [68] and the National High Blood Pressure Education Program Working Party (NHBPEP) in United States [3] require proteinuria for the diagnosis of preeclampsia. Besides, the Canadian Hypertension Society (CHS) [69] has restrained the use of the term preeclampsia and focuses more on gestational hypertension with and without proteinuria. In general, the term "proteinuric preeclampsia" is now used in several researches and clinical trials [70]. There is another discrepancy in the definition of proteinuria, since the ISSHP [68] and the ASSHP [67] define proteinuria as > 300 mg protein in a 24 h sample or a random spot protein-to-creatinine ratio of > 30 mg/mmol, while the NHBPEP [3] requires > 300 mg protein in a 24 h specimen and the CHS [69] accepts only a 24 h sample result for diagnostic purposes. Furthermore, it was demonstrated that women with proteinuric preeclampsia used more magnesium sulfate ($MgSO_4$) and had higher blood pressure at earlier gestation than non-proteinuric preeclampsia women [66]. Proteinuria is often absent in women who develop HELLP (Hemolysis, Elevated Liver enzymes, Low Platelet count) syndrome [71], but IUGR occurs equally in proteinuric or non-proteinuric preeclampsia women [66]. Moreover, the loss of serum protein leads to a decrease of intravascular volume and increased tissue edema [72]. The maternal decreased blood volume can lead to an increase in hemoglobin concentration, which is associated to an increased risk of developing IUGR [73].

Edema

In normotensive pregnancy, the amount of maternal body fluids, mostly blood, nearly doubles in order to support the growth and development of the fetus and the placenta. Edema (*e.g.* severe swelling of the face, hands and feet), caused by fluid retention, is often associated with preeclampsia. Severe edema in preeclampsia is related to proteinuria. In fact, the loss of serum protein along with increased capillary endothelial permeability leads to a decrease in intravascular volume and increased tissue edema. Loss of proteins from the blood through the urine has an osmotic attraction toward the water contained in blood and, as a consequence, the water leaks from the blood into the body's tissues [72].

As mentioned earlier, preeclampsia is a systemic disease and edema affects many organs, including liver, brain and lungs. Acute pulmonary edema is a major cause of death in women affected by preeclampsia [74] and refers to an excessive accumulation of fluid in the pulmonary interstitial and alveolar spaces [75]. During normotensive pregnancy, physiological changes in the maternal cardiovascular system, including increased plasma blood volume, cardiac output, heart rate, capillary permeability and a decrease in plasma colloid osmotic pressure, are intensified in preeclampsia and predispose women to develop pulmonary edema [76].

PATHOGENESIS: ROLE OF THE PLACENTA

Preeclampsia has been known as the "disease of theories" since the sixties [77]. Today, the precise cause of preeclampsia still remains unknown. However, extensive research has led to major advances in the comprehension of the pathogenesis of this presumably multifactorial disease. Based on those advancements, the concept of a 2 stage development of preeclampsia has been originally proposed in 1991

by Chris Redman [78]. The first stage occurs before the appearance of clinical signs (before the 20th week of pregnancy) and is characterized by a poor placentation. The second stage (after the 20th week of pregnancy) is characterized by placental stress in response to the poor placentation, leading to the maternal syndrome of preeclampsia (hypertension, proteinuria and edema). Although the 2 stage model has been revised and challenged, it remains the most widely accepted theory to explain the development of preeclampsia [42, 79-82]. It is noteworthy to mention that Drs Redman and Sargent have recently proposed that preeclampsia could be a 4 stage disease; a maternal-fetal immune maladaptation would be the cause of the poor placentation [83, 84]. This chapter will focus on the currently accepted 2 stage model but the new 4 stage theory will be discussed.

Role of the Placenta

The central role of the placenta in preeclampsia is known for more than a hundred years [85]. The development of preeclampsia is dependent on the presence of a placenta. Furthermore, the only definitive treatment for preeclampsia is the complete removal of placental tissue. It has even been shown that a fetus is not required for the development of preeclampsia, such as in molar pregnancies [86]. Moreover, in cases of preeclampsia where only the fetus has been removed, the maternal syndrome persisted until removal of the placenta [87, 88]. Even though cases of postpartum preeclampsia have been described, they have been associated with incomplete removal of placental tissue since resection of maternal syndrome has been shown after uterine curettage [89, 90].

Important pathologic changes are observed in the preeclampsia placenta, such as insufficient utero-placental blood flow leading to placental ischemia and consequently oxidative stress. In the most severe cases of preeclampsia, these pathologic changes will lead to infarcts (for a review of placental pathologic modifications in preeclampsia, see [91]). These anatomopathological modifications are not necessarily all present in cases of preeclampsia but they are significantly more frequent [92]. Moreover, these gross anatomical or histological lesions are not useful as diagnosis criteria because they are not specific to preeclampsia [93]. For example, antiphospholipid syndrom (lupus anticoagulant antibodies) presents exactly the same placental pathology as preeclampsia but without the specific maternal syndrome [94-97]. Many attempted to make correlation between the severity of these pathologic changes and the severity of the maternal syndrome but it is still disputed [98-101]. Even if no specific pathological lesions of the preeclampsia placenta have been established, the placenta is still an essential stimulus for the development of preeclampsia. Furthermore, a poor placentation is an important predisposing factor for the development of preeclampsia while placental stress is the keystone of the maternal syndrome.

Stage 1-Poor Placentation

The abnormal development of the early placenta, the insufficient invasion and remodeling of uterine spiral arteries by extravillous cytotrophoblasts, which results in reduced maternal blood supply to the placenta, is called poor placentation. The preeclampsia syndrome was considered to be caused by poor placentation [102]. However, because poor placentation also occurs in normotensive pregnancies with small fetuses, it is more likely a powerful predisposing factor for preeclampsia rather than its origin [103-105].

In normotensive pregnancies, the formation of the chorionic villi, the structural and functional unit of the placenta, is initiated at 13 days post-conception. The core of the chorionic villi is composed of the fetal stroma and the outer epithelial layer is formed by the trophoblast. The villi are classified as anchoring (or stem) villi, promoting implantation and maintenance of early pregnancy, or as floating villi, mediating placental growth and transplacental exchanges. The subtypes of trophoblast cells present in the floating villi are the villous cytotrophoblast and the syncytiotrophoblast whereas the extravillous cytotrophoblasts are located in the anchoring villi (for a review of human placental development, see [106]). The tip of the anchoring villi is formed by a column of extravillous cytotrophoblasts. The cells from layers adjacent to the fetal stroma are proliferative extravillous cytotrophoblasts. The cells from distal layers of the column have exited the cell cycle and have acquired an invasive phenotype. Invasive extravillous cytotrophoblasts (small spindle-shaped extravillous cytotrophoblasts) will spread from the distal part of the cell column into the decidua and myometrium [107]. These highly invasive extravillous cytotrophoblasts will either invade the placental bed or

terminally differentiate in large polygonal trophoblast cells [108, 109] or in multinucleated trophoblast giant cells [110]. Otherwise these cells will invade the uterine spiral arteries, acquire an endothelial-like phenotype, replace the spiral arteries endothelial cells, participate in the degradation of tunica media smooth muscle cells and terminally differentiate into endovascular extravillous cytotrophoblasts [111, 112]. The spiral arteries are thus remodeled from small and highly constricted vessels to large capacitance vessels devoid of contractile capability (Fig. **1**). This remodeling of the spiral arteries is essential to allow a proper placental perfusion to sustain fetal growth and pregnancy well-being [113].

In preeclampsia, the remodeling of uterine spiral arteries is altered. Reduced invasion of uterine spiral arteries by extravillous cytotrophoblasts in preeclampsia has been identified for the first time in 1972 [102]. Decreased number of invasive extravillous cytotrophoblasts in placental bed as well as decreased depth of invasion of these cells has been demonstrated in preeclampsia [114]. These observations correlate with the defective differentiation from proliferative to invasive extravillous cytotrophoblasts in preeclampsia [115, 116]. Moreover, increased apoptosis of extravillous cytotrophoblasts in placental bed of preeclampsia has been shown [117, 118]. Taken together, these findings support the partial invasion of the decidual segments of spiral arteries by extravillous cytotrophoblasts and the important decreased invasion of their myometrial segments [119]. In addition, spiral arteries endothelial cells are not replaced by extravillous cytotrophoblasts and smooth muscle cells are not degraded in the myometrial segments, resulting in inadequate remodeling of spiral arteries [113]. Consequently, in preeclampsia, the uterine spiral arteries remain small, contractile and high-resistance vessels, resulting in insufficient uteroplacental arterial blood flow and in poor placentation (Fig. **1**) [120, 121]. The sequence of events leading to the poor placental perfusion takes place before the 20th week of pregnancy, prior to the appearance of clinical signs. It is thus difficult to determine which mechanisms are implicated in its development. However, several factors involved in extravillous cytotrophoblasts invasion seem to be altered in preeclampsia, the most important being oxygen tension and immunologic factors.

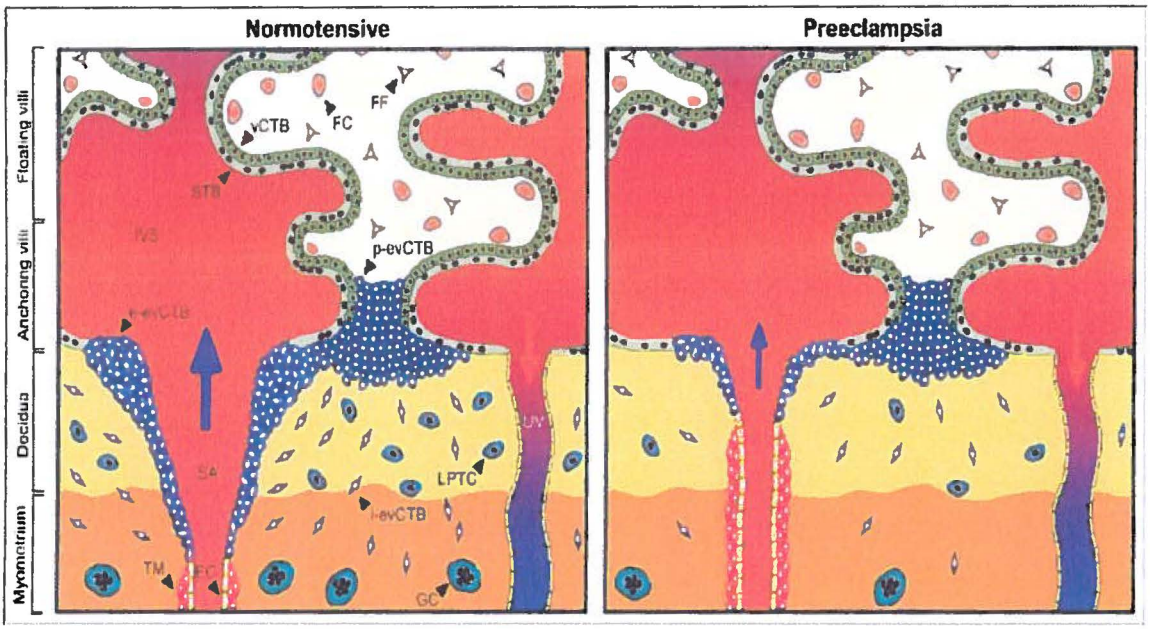

Figure 1: Placentation in normotensive and preeclamptic pregnancies. EC (endothelial cell), e-evCTB (endovascular extravillous cytotrophoblast), FC (fetal capillaries), FF (fetal fibroblast), GC (trophoblast giant cell), IVS (intervillous space), LPTC (large-polygonal trophoblast cell), i-evCTB (invasive extravillous cytotrophoblast), p-evCTB (proliferative extravillous cytotrophoblast), STB (syncytiotrophoblast), SA (uterine spiral arteries), TM (tunica media smooth muscle cell), UV (uterine vein), vCTB (villous cytotrophoblast).

Oxygen Tension

In early pregnancy, before the 10th week, there is negligible maternal blood flow to the placenta since invasive extravillous cytotrophoblasts have not yet reached and remodeled the spiral arteries. This creates a

hypoxic environment that is reduced after remodeling of the spiral arteries by invasive extravillous cytotrophoblasts. Moreover, the oxygen tension is higher in the spiral arteries than in the placental bed, creating an increasing oxygen gradient that seem to act as a stimulus for extravillous cytotrophoblasts invasion and differentiation in their endovascular phenotype [122]. *In vitro*, increased oxygen tension stimulates the differentiation of extravillous cytotrophoblasts from their proliferative to their invasive phenotype [123]. Furthermore, at higher oxygen tension, extravillous cytotrophoblasts express cellular adhesion molecules similar to those of vascular endothelial cells [124]. Accordingly, decreased oxygen tension alters extravillous cytotrophoblasts differentiation and invasion, mimicking the events taking place in preeclampsia [123-125]. In addition, the hypoxia-inducible transcription factor-1α (HIF-1α) is expressed in first trimester placental explants cultured under low oxygen and its expression is decreased under increased oxygen tension [126]. Interestingly, HIF-1α expression and activity is highly up-regulated in the preeclampsia placenta [127, 128]. HIF-1α stimulates the expression of transforming growth factor-β3 (TGF-β3), an inhibitor of villous trophoblast invasion, and the antisens inhibition of HIF-1α expression inhibits invasion and TGF-β3 expression in placental explants [129]. Taken together, these findings indicate that oxygen is a master regulator of extravillous cytotrophoblasts invasion and differentiation. However, it is currently not known if an early hypoxic placenta is the cause to the altered extravillous cytotrophoblasts differentiation and invasion or if it is the consequence of the defective remodeling of the spiral arteries.

Immunologic Factors

The fetus is an allograft, carrying both maternal and paternal antigens. The immune theory of preeclampsia proposes that a maternal-fetal immune maladaptation would result in the recognition of the extravillous cytotrophoblasts (fetal) by the decidual immune cells (maternal), causing the poor placentation [39, 130]. As mentioned earlier, a recent study by the fathers of the concept of the 2 stage disease argues that preeclampsia could be a 3 stage disease, the first step being this maternal-fetal immune maladaptation [83]. The immune theory of preeclampsia relies on many epidemiological and clinical studies. Preeclampsia occurs mostly in first pregnancies [34, 35]. It suggests that the foreign fetus triggers the maternal immune system and that it could become tolerant through successive pregnancies [131, 132]. This protective effect of multiparity is lost with a new partner [36]. Moreover, a great exposition of the maternal organism to paternal antigens, through oral and vaginal sperm exposure, will result in reduced risk of preeclampsia [36, 37]. The use of barrier contraceptives (condom or diaphragm) abolishes these protective effects [133, 134]. In addition, conception through assisted reproductive technologies, using donor sperm or surgically obtained sperm, increases the risk of preeclampsia; supporting the importance of pre-conception exposure to paternal sperm [38, 39]. Overall, these studies reinforce the concept that preeclampsia could be caused by a defective maternal immunosuppressive response to induce tolerance to paternal antigens.

The most abundant leukocytes in the decidua are uterine natural killer (uNK) cells, macrophages and T lymphocytes [135]. In the first trimester of pregnancy, 30-40% of the decidual cells are leukocytes in close contact with the invading extravillous cytotrophoblasts [135]. Invasive extravillous cytotrophoblasts expressed human leukocyte antigen (HLA)-C, which can trigger a maternal immune response since it has a paternal specificity [136]. Both uNK and T lymphocytes can recognize the HLA-C [137, 138]. Moreover, uNK are the only decidual cells expressing killer immunoglobulin-like receptors (KIR), for which HLA-C is the main ligand [137]. KIR and HLA-C are both highly polymorphic genes that are genetically inherited [139]. It is noteworthy to mention that epidemiological studies show a genetic susceptibility for both men and women born from preeclamptic pregnancies to have a child from a preeclamptic pregnancy and this risk is increased in cases of both maternal and paternal familial history of preeclampsia [140, 141]. In normal pregnancies, uNK promotes extravillous cytotrophoblasts invasion and spiral arteries remodeling by chemokines, cytokines and growth factors secretion [142]. These preeclampsia-protective effects were associated with a specific polymorphic combination of KIR and HLA-C [143]. In opposition, a specific combination of KIR and HLA-C was associated with an increased risk or preeclampsia [144]. This extravillous cytotrophoblasts-uNK recognition pattern could explain the partner-specificity and genetically inherited susceptibility for preeclampsia [83]. However, the KIR-HLA-C interaction does not clarify why preeclampsia occurs mostly in first pregnancies and why the risk decreases with subsequent pregnancies

with the same partner. This could be explained by T-cell memory. As mentioned, T-lymphocytes can recognize the HLA-C. T-lymphocytes memory seems stable enough to induce tolerance to paternal antigens in a second pregnancy [145]. In addition, NK cells and macrophages, both localized in the decidua, can elicit a form of immune memory [146-148]. As mentioned by Redman and Sargent, the greater risk of preeclampsia in first pregnancies, partner-specificity and genetic susceptibility could be explained by their novel immune theory of a 4 stage disease, although further experiments are required [83]. For more detailed review in immunology and preeclampsia see Chapter 5 from Saito in this eBook.

Stage 2 - Placental Oxidative Stress and the Maternal Syndrome

The final stage of preeclampsia is a maternal syndrome characterized by a new appearance of hypertension and proteinuria after 20 weeks' pregnancy and resolving after delivery. According to the 2 stage model - and supported by evidence - these clinical signs are the result of a maternal systemic inflammatory stress in response to the release of various syncytiotrophoblast factors. The release of these factors is stimulated by the placental oxidative stress secondary to the poor placentation (Fig. 2).

Placental Oxidative Stress

There is an increased oxidative stress during normal pregnancy [149, 150]. Furthermore, the oxidative stress and resulting damages are significantly amplified in preeclampsia [151]. In addition, numerous placental oxidative stress biomarkers are increased in preeclampsia, such as TNF-α levels [152], HNE-modified proteins [153, 154], 8-isoprostane levels [155], superoxide radical's concentration [156] and protein carbonyls [157]. Accordingly, the placenta is a major source of oxidative stress in preeclampsia and several studies have shown that the generation of placental oxidative stress is a key event in its pathogenesis [79, 82, 151, 158-160].

The placental oxidative stress was suggested to be generated by reduced utero-placental arterial blood flow resulting from deficient remodeling of uterine spiral arteries and thus creating a chronically hypoxic placenta [113, 161]. However, as reported by Hung and Burton, chronic placental hypoxia - the placenta is starved of oxygen - does not seem to be the inducer of oxidative stress in preeclampsia for many reasons [162]. In sheep, chronic restriction of the uterine blood flow is responsible for reduced placental metabolism and significant decrease of placental and fetal weight [163]. It is not the case in preeclampsia [164, 165]. Moreover, in high altitude pregnancies with restrict oxygen supply to the placental bed, the placenta does not show signs of oxidative damage [166, 167]. The trophoblast is used to low oxygen tension. During first trimester, the placental oxygen concentration is far below the one at the third trimester until it rises around 12 weeks' [168]. Hung and Burton thus suggest that the placental oxidative stress occurring in preeclampsia might be induced by hypoxia-reoxygenation rather than by hypoxia alone [162]. In preeclampsia, remodeling of the uterine spiral arteries only occurs in the decidual segment of the spiral arteries and they remain mostly vasoactive (Fig. 1). The maternal blood flow would thus enter the intervillous space at high pressure in a pulsatile manner, exposing the placenta to arbitrarily oscillating oxygen tension [169, 170]. This is supported by studies showing that hypoxia/reoxygenation generates high levels of free radicals, namely reactive nitrogen species (RNS) and reactive oxygen species (ROS) that are present in the preeclampsia placenta [171, 172]. In addition, first trimester placental explants survive well in low oxygen condition but become stressed when the oxygen tension is raised, inducing oxidative stress and apoptosis [173-175].

ROS Generation

In preeclampsia, the predominant ROS produced by the placenta are superoxide radicals ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) [156]. They are mainly generated by two intracellular sources, the xanthine dehydrogenase/xanthine oxidase (XDH/XO) pathway and the mitochondria respiratory chain.

XDH is a key enzyme in purine catabolism, catalyzing the hydroxylation of hypoxanthine to xanthine and of xanthine to urate. However, in hypoxia, XDH is irreversibly converted to XO through protease-mediated sulfhydryl oxidation [176]. Thus, cellular ATP is catalyzed to hypoxanthine and it accumulates throughout the

hypoxic period. On reoxygenation, reintroduced oxygen, hypoxanthine and XO will combine to generate superoxide and hydrogen peroxide [177, 178]. Moreover, hypoxia upregulates XO activity, enhancing hypoxanthine build up and consequently ROS formation [179]. XDH/XO mRNA and protein expression as well as enzyme activity have been detected in normal term placenta [180, 181]. In preeclampsia placentas, increased protein expression of XDH/XO is shown compared to normal placentas and XDH/XO co-localizes with nitrotyrosine residues, a marker of oxidative stress [182]. In addition, XO activity is increased in preeclampsia placenta [183]. The release of XDH/XO in the circulation upon hypoxia/reoxygenation has been demonstrated in the liver and intestine [184, 185]. However, it is yet to be shown whether it is the case in the placenta. Nevertheless, maternal circulating levels of XO are higher in preeclampsia than in normal pregnancies, so it is likely it could originate from the placenta subsequently to hypoxia/reoxygenation [186, 187]. Finally, perfusion of normal human placentas with XO induces changes in oxidative stress and apoptosis-related genes expression similar to what is described in preeclampsia [188].

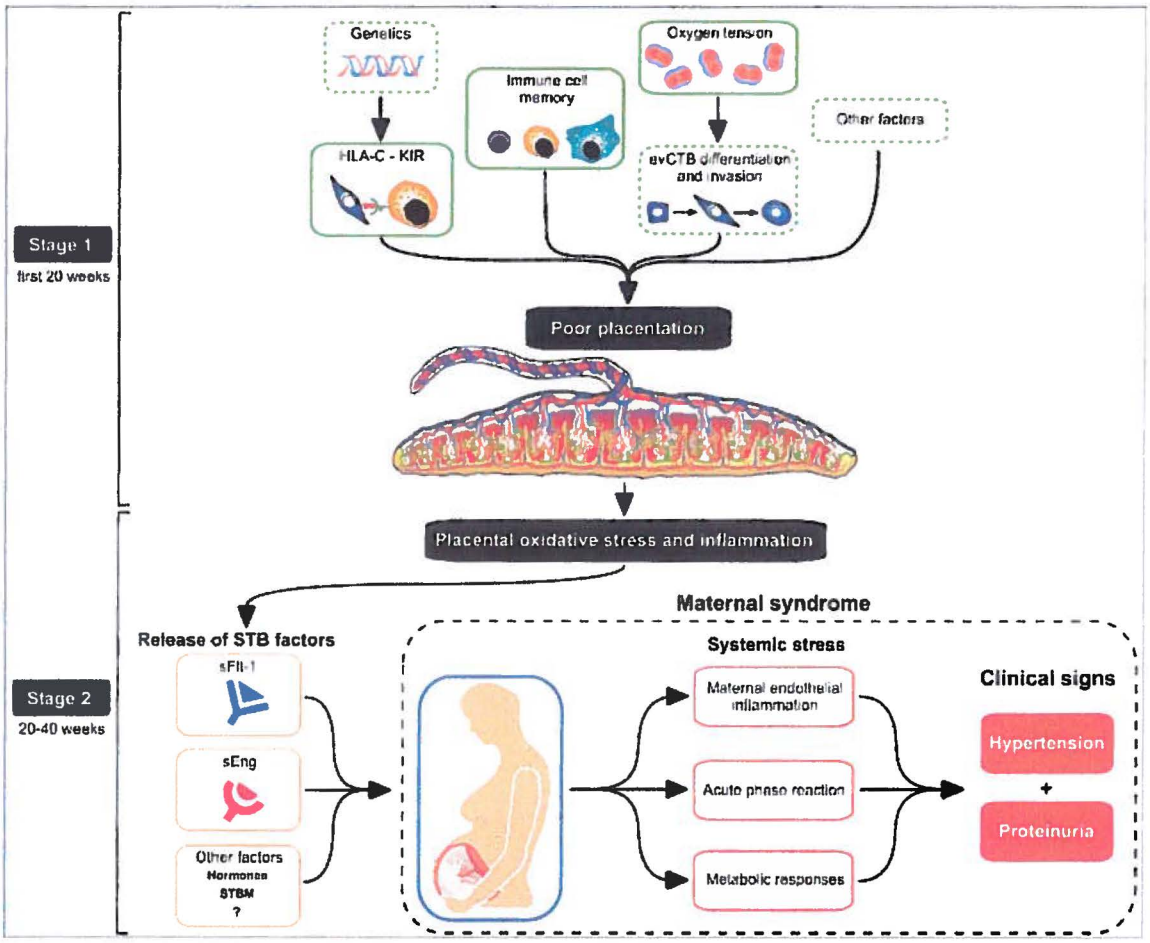

Figure 2: Summary of the pathogenesis of preeclampsia, according to the 2 stage disease theory. HLA-C (human leukocyte antigen-C), KIR (killer-cell immunoglobulin-like receptors), evCTB (extravillous cytotrophoblast), STB (syncytiotrophoblast), sFlt-1 (soluble fms-like tyrosine kinase-1), sEng (soluble endoglin), STBM (syncytiotrophoblast microparticles).

The placental mitochondria respiratory chain is another source of ROS in preeclampsia [189]. During mitochondrial oxidative phosphorylation, electrons are transferred from reduced nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) to molecular oxygen (O_2), resulting in adenosine triphosphate (ATP) synthesis [190]. The electron transfer process generates superoxide radicals [191, 192]. In normal placenta, superoxide radical's levels are tightly regulated by the manganese superoxide dismutase (Mn-SOD), in the mitochondria, and by its isoform, the copper/zinc superoxide dismutase (Cu/Zn-SOD), in the cytoplasm [193]. The SOD catalyzes the dismutation of superoxide radicals

to hydrogen peroxide and molecular oxygen [194]. However, in preeclampsia, decreased expression and activity of placental SOD are reported, resulting in increased superoxide radicals levels [160, 171, 195, 196]. The decomposition of cellular hydrogen peroxide to water and molecular oxygen is catalyzed by the catalase and the glutathione peroxidase (GPX) [197]. No difference in catalase expression and an increased catalase activity have been reported in placenta from preeclamptic compared to normal pregnancies [196]. However, the expression and activity of placental GPX are decreased in preeclampsia, reducing hydrogen peroxide elimination [160, 198-202]. Two studies have shown no difference in GPX levels between normal and preeclamptic placentas, suggesting that the GPX expression may not be correlated to its activity [203, 204]. Taken together, these findings show that preeclampsia is characterized by an increased placental ROS production and decreased placental antioxidant defense mechanisms.

Oxidative Stress Damage

Placental ROS generate broad spectrum cytotoxic effects, ranging from damaged cellular proteins and lipids to activation of cell signaling cascades leading to syncytiotrophoblast injury and ultimately cell death.

Common ROS-mediated injuries include protein carbonylation and nitrosylation as well as lipid peroxidation. Protein carbonylation is an irreversible type of protein oxidation leading to loss of protein function and to structural alterations [205]. The irreparable nature of protein carbonyls makes them markers of choice for oxidative stress damage [206]. Placentas from preeclampsia are characterized by increased levels of protein carbonyls [157, 160, 207]. Another type of oxidative-stress mediated protein alteration is the nitrosylation of protein by peroxynitrite anions. Peroxynitrite anion is the product of superoxide radical and nitric oxide interaction [208]. Peroxinitrite is a strong oxidant which, in addition to protein nitrosylation, can generates protein carbonyls, initiates lipid peroxidation, inhibits mitochondrial electron transport and nitrate tyrosine residues, thereby affecting signal transduction pathways [209-212]. Nitrotyrosine detection is used as a marker of peroxinitrite formation since they are undetectable due to their unstability [213]. Increased levels of nitrotyrosine residues are observed in placenta from preeclampsia, indicating adverse action of peroxinitrites [172, 214, 215]. Lipid peroxidation is the degradation of cellular lipids upon exposure to ROS [216]. Lipid peroxides are formed in a chain reaction which if not stop, can disturb membrane fluidity and permeability, alter ion transport, inhibit metabolic processes and injure mitochondria, inducing further ROS generation and apoptosis [217, 218]. Increased lipid peroxides have been shown in preeclamptic placentas [219-221]. An increase in lipid peroxides has been specifically demonstrated in the mitochondria and cellular membrane of the syncytiotrophoblast of preeclamptic placentas [153, 222-224]. Accordingly, syncytiotrophoblast membrane integrity alteration and mitochondrial apoptosis are observed in preeclampsia [225, 226].

ROS can regulate several cellular processes by acting as second messengers, by altering signaling pathways or by altering cellular homeostasis. In the placenta, the mitogen activated protein kinases (MAPK), the nuclear factor-κB (NF-κB) and the calcium transport are among the most significant [227, 228]. The MAPK are a family of protein kinases involved in many physiologic responses and regulatory mechanisms [229]. There are four families of MAPK, the extracellular signal-regulated kinases (ERK 1/2), the p38 kinases, the stress- activated protein kinase/c-Jun NH2-terminal kinases (SAPK/JNK) and the ERK5. ROS are known inducers of apoptosis through the p38 and SAPK/JNK pathways [230]. Hypoxia/reoxygenation activates p38 and SAPK/JNK stress pathways in villous trophoblast explants, inducing pro-inflammatory cytokines release and apoptosis [228, 231]. Moreover, increased placental activation of p38 has been reported in preeclampsia [232]. NF-κB is a dimeric transcription factor. ROS-activated NF-κB promotes the transcription of genes involved in inflammation, stress response and apoptosis, such as HIF-1α [233, 234]. Hypoxia/reoxygenation induces the NF-κB pathway in villous trophoblast explants, stimulating pro-inflammatory cytokines secretion and apoptosis [231]. Increased expression of placental NF-κB is also demonstrated in preeclampsia [235]. Calcium transport through the placenta regulates numerous cellular processes [236]. Calcium homeostasis is involved in signal transduction, neurotransmission, hormone secretion, cell-cycle regulation and mitochondrial functions [237]. Oxidative stress is a known modulator of calcium homeostasis, inducing apoptosis [238-241]. Altered placental calcium homeostasis is observed in preeclampsia [227]. A recent study suggest that altered calcium homeostasis in preeclampsia syncytiotrophoblast is secondary to oxidative stress [227]. As reviewed, ROS

activate placental signaling pathways and altere cellular homeostasis, inducing placental inflammatory stress and apoptosis in preeclampsia.

The most damaging effect of ROS is cellular death. ROS-mediated cellular death can be from necrotic or apoptotic origin. Necrosis is caused by elevated ROS levels whereas apoptosis occurs at low ROS levels [228]. As discussed previously, ROS can promote apoptosis through several ways, such as peroxynitrites and lipid peroxides generation as well as signaling cascades activation. Apoptosis is a programmed and organized, ATP-dependent, cell death occurring in normal placental development which increases throughout pregnancy [242]. Necrosis is an accidental and ATP-independent cell death not taking place in normal placental development. In normal pregnancy, apoptosis controls trophoblast differentiation, syncytial fusion and villous trophoblast turnover [243, 244]. In preeclampsia, placental apoptosis is significantly increased compared to normal pregnancy [245]. Abnormal placental apoptosis is particularly taking place in the syncytiotrophoblast [225, 245, 246]. Increased syncytial necrosis is also observed in preeclampsia [247]. Consequently, villous trophoblast turnover is altered in preeclampsia [248]. Hypoxia/reoxygenation has been demonstrated to promote syncytiotrophoblast apoptosis and necrosis as well as oxidative stress *in vitro*, mimicking changes occurring in preeclampsia [174, 231, 249]. Currently, it is not known whether these apoptotic and necrotic changes are a primary pathologic event or a secondary manifestation. The 2 stage model implies that it is a secondary event taking place after altered extravillous trophoblast invasion [78, 79, 81, 82]. However, Huppertz suggest that it is the primary placental alteration which occurs in preeclampsia [42]. Nevertheless, both hypotheses agreed that oxidative stress disrupts syncytial architecture, through increased apoptosis and necrosis, and stimulates the release of factors in the maternal blood which are responsible for the systemic inflammatory response [42, 250]. Furthermore, it has been demonstrated that H/R stimulates the release of syncytiotrophoblast pro-inflammatory factors, such as tumor necrosis factor-alpha (TNF-α), cell-free DNA, soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng) and syncytiotrophoblast microfragments (STBM) [249, 251-254].

Release of Syncytiotrophoblast Factors

In preeclampsia, oxidative stress disrupts syncytial architecture and promotes the release of pro-inflammatory factors (Fig. **2**). These syncytiotrophoblast factors include activin-A [255], cell-free fetal DNA [256], corticotrophin releasing hormone (CRH) [257], leptin [258] and TNF-α [152]. The most studied are the STBM [259], the sFlt-1 [254] and the sEng [253]. STBM are syncytial membranes shed into the maternal circulation that impair maternal endothelial cell functions and stimulate inflammation [226, 259-262]. Their levels in maternal blood are correlated with the severity of preeclampsia [263]. In preeclampsia, sFlt-1, a circulating antagonist of vascular endothelial growth factor A (VEGF-A), binds and inactivates VEGF and placental growth factor (PlGF), inducing systemic endothelial dysfunction [254]. Levels of sFlt-1 are elevated in patients with preeclampsia 2 to 5 weeks before onset of clinical symptoms [264-266]. Moreover, sFlt-1 levels are associated with the severity of the syndrome [264, 267]. The expression of sFlt-1 is regulated by HIF-1α [268]. Hypoxia/reoxygenation-induced oxidative stress increased HIF-1α and sFlt-1 expression in placental explants by a p38 and a NF-κB dependent pathway [228, 269]. sEng, a transforming growth factor-beta (TGF-β) co-receptor, interferes with TGF-β signaling, disrupting vascular homeostasis and inducing hypertension *in vivo* [253]. As for sFlt-1, sEng levels are elevated weeks before onset of clinical symptoms in patients with preeclampsia [270]. sEng expression is also regulated by HIF-1α and correlates with disease severity [253, 271]. Redman and Sargent propose that sFlt-1 and sEng are as much upregulated by hypoxia than by placental inflammatory stress since HIF-1α has been shown to be regulated by both [79]. Thus, the cocktail of pro-inflammatory syncytial factors will contribute to the development of the maternal systemic inflammatory response.

Systemic Inflammatory Response

Preeclampsia is often described as an endothelial disorder. It is true that sFlt-1 and s-Eng are powerful anti-angiogenic factors and probably constitute the primary cause of the endothelial dysfunction associated with the clinical signs of preeclampsia. Although, endothelium inflammation is involved in preeclampsia, the stress response is mostly systemic and involves other components of the inflammatory network such as acute-phase response and metabolic responses [79] (Fig. **2**).

The clinical feature of preeclampsia is new onset of maternal hypertension and proteinuria resolving after delivery. The maternal hypertension results from diffuse endothelial dysfunction and the proteinuria is ascribed to glomerular endotheliosis [272, 273]. There is an increased expression of endothelial activation and endothelial dysfunction markers in preeclamptic women, including von Willebrand Factor [274], endothelin [275], vascular cell adhesion molecule (VCAM) [276], thrombomodulin [277], platelet-derived growth factor (PDGF) [278], cellular fibronectin [279] and soluble E-selectin [280]. The incubation of serum from preeclamptic women with endothelial cells results in endothelial dysfunction, suggesting that circulating factors could be responsible for the hypertension and proteinuria [253]. Therefore, in pregnant rats, elevated levels of sFlt-1 cause preeclampsia-like symptoms, including hypertension, proteinuria and glomerular endotheliosis [254]. Moreover, sEng administration amplifies sFlt-1-mediated endothelial damage in rats [253]. sFlt-1 and sEng could thus be responsible for the maternal endothelial dysfunction and the clinical feature of preeclampsia [273].

In addition to endothelium inflammation, preeclampsia is characterized by an acute-phase reaction [79, 281]. The acute-phase reaction is a complex endocrine and metabolic response triggered by a number of inflammatory cytokines mainly secreted by macrophages and monocytes at the inflammatory site [282, 283]. These cytokines stimulate the production of acute-phase proteins by hepatocytes. Acute-phase proteins are defined as positive when their plasma concentration increases and negative when it decreases by at least 25% in reaction to inflammatory disorder [284]. In preeclampsia, positive acute-phase response proteins include c-reactive protein [285], plasminogen [286], angiotensinogen [287], many proteins of the complement system [288-290] and several clotting factors [291, 292]. Negative acute-phase response proteins consist of C56 complement protein [293] and albumin [294].

Finally, various metabolic responses are activated secondary to the systemic inflammation in preeclampsia; supporting the proposition that preeclampsia is not just an endothelial dysfunction [79]. They mainly involve lipid metabolism. Hyperlipidemia is a feature of normal pregnancy but it is significantly increased in preeclampsia before onset of maternal symptoms [295]. Hypertriglyceridemia, insulin resistance, increased circulating level of free fatty acids, small density lipoproteins, and occurrence of oxidized low density lipoproteins characterize preeclampsia [296-298]. TNF-α and other pro-inflammatory factors are known inducers of hyperlipidemia, indicating that it may be mediated by syncytial factors [299, 300]. In addition to be a risk factor, obesity increases the inflammatory response in preeclampsia [23-25, 301, 302]. Adipocytes secrete numerous pro-inflammatory cytokines, including TNF-α and leptin [303, 304]. Leptin is a powerful pro- inflammatory stimulus and its secretion is significantly increased in preeclampsia [305-307].

The involvement of multiple components of the inflammatory network in the pathology of preeclampsia points toward a more systemic response than just an endothelial dysfunction.

PREDICTION, PREVENTION AND TREATMENTS

The most effective treatment for preeclampsia is delivery itself [3, 44], but it may not be possible for a premature fetus, particularly if the mother has mild disease [3]. Several factors are considered to determine the best time to deliver, including the severity of the condition, the risk of complications, how badly the fetus is affected and the chances of survival or of a premature baby. Clear indications for delivery are: severe IUGR, alarming fetal surveillance or oligohydramnios (i.e deficiency in amniotic fluid), gestational age of 38 weeks or more, maternal platelet count below 200 x 10^9/L, maternal progressive deterioration of hepatic or renal functions, placental abruption presumption and eclampsia [3]. In general, the later the baby is born, the better, with the exception of severe preeclampsia if the baby grows very inadequately because of the poorly functioning placenta.

In general, vaginal delivery is preferable, in order to avoid extra stress of cesarean delivery. In the latter case, the use of regional anesthesia is preferred because it involves less maternal risk, except in the presence of coagulopathy, where the use of regional anesthesia is generally contraindicated [3]. During labor, efforts are made to prevent seizures and to control hypertension [44]. Until the baby is born, preeclampsia symptoms can be managed and several randomized trials reported the effective use of various

methods to reduce the rate or severity of preeclampsia [308]. Methods used to prevent or treat preeclampsia are resumed in Table 2.

Table 2: Possible prevention and treatment of preeclampsia

Anti-hypertensive drugs [309, 310]
Low-dose aspirin (anti-platelet agent) [311, 319]
Low-molecular-weight heparin (LMWH) (anti-coagulant) [312, 313]
Calcium supplementation [60, 62]
Antioxidant [79, 82, 173]
Exercise [314, 315]

Treatments

Magnesium Sulfate ($MgSO_4$) and other Antihypertensive Drugs

$MgSO_4$ is an antihypertensive agent that helps to prevent eclamptic seizures in pregnant women [316, 317]. Antihypertensive drugs are given to handle or prevent worsening of the symptoms and can thus temporize over the short term to allow safe delivery with a more mature fetus [318]. $MgSO_4$ is superior to phenytoin (Dilantin) and diazepam (Valium) for the treatment of eclamptic seizures. Usually, a 6 g dose of $MgSO_4$, followed by a continuous infusion at a rate of 2 g/h is applied [316, 317]. Although $MgSO_4$ is commonly used, there is yet no study demonstrating its preventive action on preeclampsia [309, 310].

Other antihypertensive drugs are commonly used in the treatment of severe preeclampsia. The aim of antihypertensive therapy is to prevent maternal cerebrovascular complications and to lower gradually systolic pressure to 140-155 mm Hg and diastolic pressure to 90-105 mm Hg [3]. The medical treatment should be initiated in hospitals. Antihypertensive agents are indicated at a SBP of ≥ 170 mm Hg or at a DBP of ≥110 mm Hg. For high-risk women, a threshold of 160/100 mm Hg is appropriate. These measures are consistent with international guidelines [44]. Note that a rise in SBP to ≥ 160 mm Hg is more related to development of strokes in preeclampsia than a rise in DBP to ≥110 mm Hg [319]. Hydralazine (Apresoline) and labetalol (Normody, Trandate) are most commonly used, but the latter should not be used in women with asthma or congestive heart failure.

Alternative treatments exist, such as nifedipine (Procardia), a dihydropyridine calcium antagonist, and sodium nitroprusside (Nitropress), a nitric oxide donor/releaser and potent vasodilator, but significant risks are associated with their use. Use of angiotensin-converting enzyme inhibitors is contraindicated in pregnant women [3]. Usually, blood pressure normalizes after delivery but a diagnosis of chronic hypertension is made if it remains elevated at 12 weeks postpartum [3]. Some studies have shown that the use of long-acting oral antihypertensive agents in mild preeclampsia, may lead to IUGR [320, 321]. In addition, abrupt drops in blood pressure should be avoided [322].

Prevention

Many measures have been suggested to prevent preeclampsia but none are well-established [26, 44]. These measures include prophylaxis as low-dose aspirin, low molecular weight heparin, calcium supplementation, and antioxidant use.

Low-Dose Aspirin (Anti-Platelet Agent)

Prophylaxis with aspirin has been investigated in a number of studies. The underlying principle is that preeclampsia is characterized by an imbalance between vasoconstrictive and vasodilating prostaglandins, with an excess of the vasoconstrictive thromboxane. Preeclampsia involves an imbalance between prostacyclin (PGI2), an anticoagulant and vasodilator prostaglandin, and thromboxane A2 (TXA2), a pro-coagulant and vasoconstrictor prostaglandin. Aspirin, which is an inhibitor of cyclooxygenases (COX), reduces TXA2, amends the report PGI2/TXA2 and therefore tends to reestablish the physiological balance

[311]. It has been shown that low-dose aspirin inhibits thromboxane overproduction induced by preeclampsia, but has no effect on vascular prostacyclin production [323].

Some evidences support the use of low-dose aspirin in certain high-risk women, like in women with abnormal uterine artery on Doppler ultrasound examination performed in the second trimester [324]. On the other hand, aspirin therapy does not seem to be generally beneficial for pregnant women [3, 44]. Other studies observed that oral aspirin 75 to 150 mg/day reduces by 10% [325] to 17% [323] the rate of preeclampsia and by 9% [325] to 14% [323] the rate of neonatal mortality. In 2007, a meta-analysis conducted using individual data from all trials on low-dose aspirin study against placebo during pregnancy, brought together 32 217 patients [325]. Overall, results confirmed that aspirin was effective in the prevention of preeclampsia with a decreased risk of about 10%. Treatment should be started between 12 and 14 gestational weeks with a dose between 75 and 160 mg/day. Two randomized studies showed taken at bedtime, aspirin could achieve lower blood pressure values [326, 327]. Despite the large number of studies and patients involved, it remains difficult to define precisely the groups of patients for whom this treatment is indicated.

Low-Molecular-Weight Heparin (LMWH) (Anti-Coagulant)

Low-molecular-weight heparin has been proposed in the prevention of preeclampsia. In reality, there is no evidence that this treatment is effective. After a first pregnancy marked by a vascular complication, several authors have shown that the rate of preeclampsia was reduced during the next pregnancy when treated with LMWH [312, 313]. In a recent randomized trial, the use of LMWH (deltaparin, weight adjusted, 4000-6000 IU/day, on or before the 16th to 36th gestational week) lowered the incidence of preeclampsia from 23.6% to 5.5% in women with previous preeclampsia or IUGR [328]. Nevertheless, the level of evidence of these studies is questionable, in part because of the low recruitment [311].

Calcium Supplementation

Several studies have shown that calcium supplementation reduces blood pressure in pregnant women [59] at risk for hypertensive disorders or with low dietary calcium intake [60-62]. Calcium supplements probably act by relaxing either parathyroid hormone or renin release, decreasing intracellular calcium in vascular smooth muscle and consequently its contractility [60, 329]. Thus, calcium supplementation could reduce preterm labor and delivery if it reduces uterine smooth muscle contractility [330]. One of the hypotheses that could explain this relationship is that lack of calcium stimulates parathyroid hormones and release of renin, which led to an increase of intracellular calcium in smooth muscle cells and therefore to vasoconstriction. Thus, intake of calcium may also have an indirect effect on smooth muscle cells functions. Results of many studies led to recommend a calcium intake of at least 1.5 g/day, starting at 15 gestational weeks and maintained throughout pregnancy in populations with a basic calcium intake of 600 mg/day, especially in patients at high risk of preeclampsia [60]. A recent study observed that calcium homeostasis was perturbed in preeclamptic primary syncytiotrophoblast cells. In addition, they observed that the expression of many important genes for the trans-placental transfer of calcium was decreased in preeclamptic placentas. They concluded that an excess of oxidative stress and a lack of ATP level could be the cause of these perturbations [227].

Antioxidants

As discussed above, it has been proposed that oxidative stress could be a causative mechanism of the systemic inflammatory response in preeclampsia. Likewise, studies have demonstrated higher level of oxidative stress in placentas from preeclampsia pregnancies [79, 82, 151, 158-160]. Consequently, antioxidant therapy has been projected. Vitamin C is a dietary scavenger of free radicals acting in liquid phase, while vitamin E acts *in vivo* by preventing the formation of lipid peroxides. These vitamins thus protect enzymes, proteins and cells from destruction caused by oxidative stress. Preeclampsia is associated with a decrease of these antioxidants which are essential in maintaining the defenses of the organism facing oxidative stress [311]. Trials do not support the administration of vitamin C and E to prevent preeclampsia and proposed that massive and expensive trials should ceased until further research is undertaken [331-

336]. They demonstrated that the combination of 1000 mg of vitamin C and 400 IU of vitamin E did not reduce the risk of preeclampsia. A proposed explanation for the failure of vitamins to prevent or ameliorate preeclampsia is the inadequate dosage [336]. Extrapolation from animal models suggested that 10-15 g of vitamin C is required to reduce oxidative stress in humans [336].

Moreover, among its many benefits, regular physical activity has shown to enhance endogenous antioxidant defenses, which may reduce the risk of preeclampsia [314, 315]. A randomized clinical trial comparing walking versus stretching on the incidence of preeclampsia has observed that the incidence of preeclampsia was of 14.6% among walkers and of 2.6% among stretchers. Moreover, the mean level of transferrin, an antioxidant marker, was significantly higher in the stretching group. They conclude that regular stretching exercises may promote endogenous antioxidants among women at risk for preeclampsia [337]. Still, further researches with larger sample size are needed to determine optimal timing, intensity and other types of exercises that could be beneficial for pregnant woman. Antioxidant therapy is not currently undergoing but research on this alternative is promising. Novel approaches to prevent through amelioration of the oxidative stress include melatonin supplements, selenium supplements, antiperoxinitries strategies and statins [338, 339].

MATERNAL OUTCOMES

Women affected by preeclampsia who do not receive prenatal care are 7 times more likely to die from complications related to preeclampsia [350]. Black women seem to be 3 times more affected by maternal death than Caucasians. Precise reasons of racial differences remain elusive, but disparities in health status and access or quality of prenatal care [350] and vitamin D deficiency [351] are proposed.

Women who suffer from severe preeclampsia may develop serious headaches, visual blurring, acute liver pain, seizures and/or eclampsia in 0.1% of all pregnancies [340, 341], severe proteinuria from renal failure, hemolysis, thrombocytopenia, HELLP syndrome in 0.17 to 0.8% of all live births or kidney damage [342]. Besides maternal complications, the fetus can also be distressed. For instance, preeclampsia leads to IUGR in 30% of cases [308]. As mentioned earlier, proteinuria is not universally considered for the diagnosis of preeclampsia [66]. It was observed that in up to 20% of eclampsia cases [343, 344] and 5% to 15% of HELLP syndrome cases [345], proteinuria may not be present. This section will give details about brain, liver and kidney damage [103, 340], in addition to some fetal outcomes, in particular IUGR. Most common maternal and perinatal outcomes are listed in Table 3.

Table 3: Maternal and perinatal outcomes

Eclampsia (2%)
HELLP syndrome
Kidney damage
IUGR

Eclampsia

Eclampsia complicates about 2% of preeclampsia cases [317] and is defined as the occurrence of seizures. Severe headaches or visual blurring are common signs of its onset. In general, eclamptic seizures occur after the development of hypertension and proteinuria [344], but in 20% of cases, proteinuria is absent [344]. It should also be noted that in 10-15% of cases of eclampsia, blood pressure is normal [346]

Postpartum (48 h to one month after delivery) eclamptic seizures occurs in 28% of cases [341]. Although it has been theorized that eclampsia was the linear evolvement of preeclampsia, more than one-third of women with postpartum eclampsia do not manifest signs of preeclampsia [347]. A retrospective analysis showed that eclampsia was not an evolution from preeclampsia [348].

Prevention and treatment of eclampsia is facilitated by the use of $MgSO_4$ [349], which is the anticonvulsant of choice, inexpensive and clearly more effective than others treatments [350-352]. Unfortunately, $MgSO_4$

is not available in all developing countries, characterized as system and market failures for several people [353, 354].

HELLP Syndrome

In normotensive pregnancies, the maternal blood volume expended and the platelet count can fall below 200 x 10^9/L. Probably due to an increased consumption and intravascular destruction, the platelet count can fall further in preeclamptic pregnancies [355]. Hemolysis, HELLP syndrome can be, in 10-20% of cases [308], a severe deviation of preeclampsia and may lead to immediate delivery to prevent development of precarious thrombocytopenia or hemolysis [356]. In about 15% of HELLP syndromes cases, patients do not present symptoms, or complain about upper right quadrant and/or epigastric pain, nausea or vomiting [357]. Women with HELLP syndrome might need intensive care because of the possible complications of hepatic encephalopathy, acute renal dysfunction, hepatic rupture and bleeding [357]. The presence of HELLP syndrome is an indication for delivery, in order to prevent detrimental thrombocytopenia or hemolysis [356]. Maternal mortality rates due to HELLP syndrome range from 1% in the US [358] to 30% in less developed countries [359].

Generally, this syndrome arises in the 2nd or 3rd trimester and the liver's condition normalizes within 2 weeks after delivery [357], but 7-30% of preeclampsia cases developed HELLP syndrome postpartum [360]. Multiparity, advanced maternal age and white ethnic origin are known risk factors [342]. In women who already experience HELLP syndrome, the risk of recurrence in subsequent pregnancies is increased [361, 362].

Endothelial dysfunction is considered central in systemic disease like PE and HELLP is characterized by microangiopathic hemolysis, which suggests endothelial damage. In many cases, HELLP is accompanied by eclampsia. It was reported that the incidence of HELLP syndrome after diagnosis of eclampsia ranges from 10.8% to 32.1% [363, 364]. Moreover, the incidence of eclampsia after HELLP syndrome diagnosis ranges from 6% to 52% [358, 365]. In presence of HELLP syndrome, endothelial dysfunction may injured blood-brain barrier and contributed to higher blood pressure in the cerebral area [319] and increased risk of dispersed intravascular coagulation which may contribute to cerebral hemorrhage. It was demonstrated that the majority of women with multiple seizures had HELLP syndrome [366]. HELLP must be described as a systemic disorder, involving the lungs [367], liver [368-370], central nervous system [368, 371]and kidney damage [359, 368].

Treatment with diuretics are not recommended because they can cause utero-placental hypoperfusion [372]. On the other hand, intravenous $MgSO_4$ with platelet, coagulation support, or both, are recommended, in particular in the presence of bleeding. If gestational age is less than 34 weeks, corticosteroids should be taken in order to promote fetal lung maturity, with no maternal benefits [373].

Kidney Damage

The kidney participates directly in the regulation of blood pressure. During normotensive pregnancies, a number of renal physiological adaptations are implemented and their sudden or progressive disappearance is part of the pathophysiological process observed in preeclampsia [374]. Renal failure during preeclampsia is defined by creatinine concentrations exceeding 90 mmol/L and urea concentrations superior to 7 mmol/L [374]. Acute renal failure may occur in about 5% severe cases of preeclampsia [308, 374], frequently complicated by pulmonary edema.

Kidneys pathologic analysis of women that suffer from preeclampsia may show infarction, necrosis and intraparenchymal hemorrhage of adrenal glands [375]. Structural changes in renal glomeruli, as vacuolization and swelling of the endothelial cells, as well as loss of the capillary space are described by the term glomerular endotheliosis, which is accompanied by subendothelial deposits of fibrin, decreasing the surface area for filtration [376]. As mentioned earlier, glomerular endotheliosis is associated with the proteinuria observed in preeclampsia cases. In some cases, trace to mild glomerular endotheliosis may occur at term in normotensive pregnancies [340].

Podocyturia, defined as urinary excretion of viable podocytes (glomerular epithelial cells), was recently observed in women with preeclampsia [377]. It was demonstrated that podocyte damage and detachment have a role in the development of proteinuria, but podocyturia seems to be confined to active disease only, when proteinuria can be present also during chronic phases of glomerular damage [378, 379]. Garovic *et al.* [377] suggested that podocyturia may contribute to proteinuria in preeclampsia since podocytes have a very restricted regenerative capacity. As a consequence, podocytes loss may lead to a disruption of the glomerular filtration barrier, generating proteinuria. On the other hand, normotensive pregnant women and women with hypertension or proteinuria in the absence of preeclampsia did not have podocyturia. Hence, podocyturia is not a direct result of all hypertensive kidney damage or a marker of proteinuria, but it appears to be a sensitive marker of renal damage and proteinuria in preeclampsia, where a positive correlation between the degree of proteinuria and podocyturia was observed [377].

Fetal Outcomes

IUGR

Besides the fact that maternal health can be affected by severe preeclampsia, fetal condition can also be altered. Fetal and neonatal complications include iatrogenic prematurity (i.e delivery intended by the doctor given the critical condition of the mother), oligohydramnios, increased risk of perinatal death and IUGR [308].

IUGR complicates about 10% to 25% of severe preeclampsia cases [308]. Similar to preeclampsia, IUGR usually appears in the 2nd or 3rd trimester of pregnancy, its underlying pathology taking place in the 1st trimester [113]. Although often interchanged, terms IUGR and "small for gestational age" (SGA) do not refer to the same condition. IUGR refers to a fetus that is at risk for adverse perinatal morbidity and mortality, while SGA fetuses can be just constitutionally small. Therefore, IUGR refers to SGA fetuses who display other signs of chronic hypoxia or malnutrition [380]. The American College of Obstetricians and Gynecologists (ACOG) suspects IUGR when fetuses have an estimated weight below the 10th percentile [44].

Preeclampsia and IUGR are pregnancy specific disorders sharing characteristics such as an abnormal placental implantation, a marked proliferation of villous cytotrophoblastic cells and a focal necrosis of the syncytiotrophoblast [381, 382], placental malperfusion secondary to abnormal implantation and deficient maternal spiral artery conversion [383, 384], decreased intrauterine artery blood flow leading to abnormal placentation and consequently decreased supply to the developing fetus [384]. The decreased blood volume seen in preeclampsia can lead to an increased in maternal hemoglobin concentration and is associated with an increases risk of IUGR [355].

A recent study provides strong evidences for the relation between preeclampsia and IUGR [382] but others have different hypotheses. Newhouse *et al.* [384] suggested that normotensive IUGR and IUGR with preeclampsia are two distinct pathologies with a unique impact on trophoblast function. Mari *et al.* [385] demonstrated that fetal cardiovascular changes (Doppler wavelength) observed in patients with preeclampsia differed from those seen in normotensive IUGR patients. Lorenzi *et al.* [381] described preeclampsia as a maternal syndrome, while IUGR affects mostly the fetus, suggesting that preeclampsia and preeclampsia associated with IUGR could be considered as two pathologies with different origins (maternal/placental-fetal). Huppertz proposed that not the same trophoblast cells would be altered in PE and IUGR [42]. PE would be caused by altered release of villous trophoblast factors while IUGR would result from failure of extravillous trophoblast to transform maternal spiral arteries. This however remains to be shown. A recent study showed evidences of greater maternal vascular compromise of the placental in preeclampsia cases compared to IUGR [383]. However, a recent review exposed links between those pathologies [386]. A few years ago, Rasmussen *et al.* [382] demonstrated that women who have had a growth-restricted infant without preeclampsia are more likely to have preeclampsia in subsequent pregnancies. This indicates the similarity between IUGR and preeclampsia. It was also shown that critical maternal complications are more frequent in preeclampsia patients with IUGR compared to preeclampsia patients without IUGR; the smaller the fetus, the more severe the maternal complications [387].

In general, IUGR appear as a complication of severe preeclampsia but in some cases, preeclampsia is described as an outcome of IUGR. In fact, it was reported that patients with IUGR tend to have higher blood pressure than women with normal growth fetuses [388] and that elevated maternal blood pressure is associated with decreased birth weight in normotensive pregnancies [389]. Results obtained from Tranquilli and Giannubilo suggest that IUGR is a step toward preeclampsia [388]. These two conditions may be very closely linked [390]. IUGR has been considered one of the symptoms resulting from impaired uteroplacental blood flow and it was suggested that preeclampsia should be considered as the clinical syndrome [308]. Additionally, as in preeclampsia, it has recently been suggested that the use of 1st trimester biochemical markers in combination with Doppler screening is promising for the early detection of IUGR [391].

Growth restriction is now documented as a major risk factor for premature atherosclerosis [392]. In addition, IUGR has been associated with low bone mass in infancy and increased risk for osteoporosis development in the adult [393]. With respect to maternal-fetal transfer of nutrients, there is growing evidence that limited intrauterine growth due to inadequate maternal-fetal nutritional exchange is associated with lower bone mass in infants [394], children [395] and elderly men [396].

CONCLUSION

In conclusion, concerning long-term prognosis, women with severe preeclampsia have significant higher risks of developing cardiovascular disease afterwards. A meta-analysis has observed a relative risk of 3.7 for hypertension (14 years of follow up), 2.16 for ischemic heart disease (11.7 years), 1.81 for stroke (10.4 years) and 1.49 for maternal death (14.5 years) [397].

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