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Impact of the COVID-19 pandemic on Montreal municipal wastewater leading to increase exposure to endocrine disruptor nanoparticles: potential effect on placental biology.

By

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ABSTRACT

The COVID-19 pandemic brought about a sudden change in behavior, leading to increased exposure to harmful compounds derived from single-use plastics, and environmental impacts whose effects may be felt for generations. To this end, we analyzed Montreal municipal wastewater data for levels of suspended solids and oxidizing species. Our analyses show that human activity in times of sanitary restriction leads to a temporary reduction in wastewater contamination. In the second part of this study, we focused on metal nanoparticles as emerging compounds linked to single-use plastics. Specifically, we focused on titanium dioxide, zinc oxide and cerium dioxide. We tested their effects on JEG-3 choriocarcinoma cells (placental cell model). First, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assays were used to determine the IC50 concentration of each of the nanoparticles analyzed. We subsequently performed flow cytometry and immunofluorescence analyses to determine the apoptotic state and condition of cell nuclei, respectively. Our results show that all nanoparticles reduce cell viability and increase apoptosis. The greatest effects were observed with zinc oxide, which showed an IC50 of 153.2 and 146.5 ug/ml at 24hr and 48hr exposure respectively. Overall, our results indicate that nanoparticles have a deleterious effect on the viability and proliferation of JEG-3 cells, a placental cell model, suggesting a potential effect of these three nanoparticles on pregnancy and fetal development. Further studies on primary placental cell cultures are required to confirm and extend these findings.

Keywords: COVID-19, wastewater, Choriocarcinoma cells JEG-3, nanoparticles, titanium dioxide, zinc oxide, cerium dioxide, viability, placenta

RÉSUMÉ

La pandémie de COVID-19 a entraîné un changement soudain des comportements qui a conduit à une exposition accrue aux composés nocifs dérivés des plastiques à usage unique ainsi qu'à des impacts environnementaux dont les effets peuvent se faire sentir pendant des générations. À cette fin, nous avons analysé les données relatives aux eaux usées municipales de Montréal pour déterminer les niveaux de matières en suspension ainsi que les espèces oxydantes. Nos analysent démontrent que l'activité humaine en temps de restriction sanitaire entraîne une réduction temporaire de la contamination des eaux usées. Dans la seconde partie de cette étude nous nous sommes concentrés sur les nanoparticules métalliques en tant que composés émergents liés aux plastiques non réutilisables. Plus particulièrement, nous nous sommes concentrés sur le dioxyde de titane, l'oxyde de zinc et le dioxyde de cérium. Nous avons testé leurs effets sur les cellules de choriocarcinome JEG-3 (modèle de cellules placentaires). Dans un premier temps, les tests de viabilité cellulaire MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) ont été effectués pour déterminer la concentration IC50 de chacune des nanoparticules analysées. Nous avons par la suite effectué des analyses de cytométrie en flux et d'immunofluorescence, pour déterminer l'état apoptotique et la condition des noyaux des cellules, respectivement. Nos résultats montrent que toutes les nanoparticules réduisent la viabilité cellulaire et augmentent l'apoptose. Les effets les plus importantes ont été observé avec le ZnO, qui montrent un IC50 de 153.2 et 146.5 ug/ml à 24hr et 48hr d'exposition respectivement. Dans l'ensemble, nos résultats indiquent que les nanoparticules ont un effet délétère sur la viabilité et la prolifération des cellules JEG-3, un modèle de cellules placentaires, suggérant un effet potentiel de ces trois nanoparticules sur la grossesse et le développement du fœtus. D'autres études devront être menées sur les cultures cellulaires placentaires primaires pour confirmer et approfondir ces résultats.

Keywords : COVID-19, cellule de choriocarcinome, JEG-3, eau usée, nanoparticules, dioxyde de titane, oxyde de zinc, dioxyde de cérium, viabilité, placenta

SYNOPSIS (FRENCH)

I. Résumé des Connaissances

a. La pandémie de COVID-19 et ses impacts environnementaux

Le virus du SARS-CoV-2 a émergé à la fin de 2019 et s'est rapidement propagé à travers le monde et une pandémie à été déclarée par l'Organisation mondiale de la santé (OMS) le 13 mars 2020. Le virus SARS-CoV-2 qui cause la COVID-19 a émergé à Wuhan en Chine par transmission zoonotique à partir des chauves-souris. Le premier cas au Canada a été détecté le 25 janvier 2020 et le 13 mars 2020 l'état d'urgence sanitaire a été déclaré successivement dans toutes les provinces et tous les territoires canadiens, en commençant par le Québec et se terminant par le Yukon le 27 mars. Plus spécifiquement au Québec, le premier confinement majeur a débuté le 13 mars 2020 et c'est terminé le 27 mai 2020 dans la région de Montréal. La deuxième fermeture à Montréal s'est déroulée du 1er octobre 2020 jusqu'au 25 mai 2021. Enfin, un dernier confinement a eu lieu du 20 décembre 2021 au 12 mars 2022 (Table 1.1; figure 3.2). La pandémie de la COVID-19 a causé des changements drastiques dans les habitudes de vie de la population générale, entre autres dans l'utilisation des produits sanitaires, en particulier une augmentation dans la fréquence et la quantité d'usage. Ce qui a entraîné une augmentation d'exposition et de mauvaise utilisation de ces produits causant un risque accru d'irritation et d'empoisonnement même avec des produits relativement non toxiques. En outre, il y a eu une augmentation de l'usage des plastiques dans tous les secteurs et de l'utilisation d'équipement de protection individuelle (masques, gants) dans la population générale et le secteur médical. Cet usage accru a concouru à augmenter les taux de pollution causés par l'élimination inappropriée de ces items. Les plastiques et les gants libèrent des contaminants comme les microplastiques et le bisphénol A (BPA) qui peuvent être toxique directement ou via bioaccumulation, souvent, les effets pourraient ne pas être immédiatement apparents. D'autre part, la pandémie a aussi causé une réduction de l'activité humaine qui a conduit à une amélioration de la qualité de l'air et de l'eau, et même une restauration de la vie aquatique.

En première partie, le présent projet se concentre sur les effets de la pandémie de la COVID-19 sur l'eau, spécifiquement les eaux usées dans la région de Montréal. Une étude en Malaisie a mesuré le « Water Quality Index » (table 1.2) des rivières et a démontré que la qualité de l'eau s'était améliorée d'un niveau (niveau-II à niveau-I) en mars 2020. Similairement, une étude sur des rivières transfrontalières entre le Bhoutan, l'Inde et le Bangladesh a montré qu'il y avait une période d'amélioration de qualité d'eau lors des périodes de confinements causées par la

pandémie de la COVID-19, et que le niveau de contamination était souvent accru après la levée des confinements. Dans tous les cas, l'augmentation d'exposition aux produits de nettoyage et aux plastiques a introduit de nouveaux contaminants ou aggravé les effets de ceux existants. Deux exemples de tels contaminants sont le BPA mentionné ci-dessus et les composés d'ammonium quaternaire. Le BPA est principalement utilisé dans la production des plastiques et a été étudié depuis longtemps pour ses effets xénœstrogène, c'est-à-dire qu'il est capable d'imiter l'estrogène, soit de se lier aux récepteurs d'estrogène et simuler les effets des estrogènes. Les composés d'ammonium quaternaire sont présents dans les produits de nettoyage et des études ont démontré qu'ils peuvent réduire la fertilité des souris mâles et femelles. Les composés comme le BPA et les composés d'ammonium quaternaire sont classifiés comme des perturbateurs endocriniens, comme leur nom le suggère, ils interfèrent avec le système endocrinien (hormonal) du corps humain. La pandémie de la COVID-19 a donc conduit à une augmentation d'exposition aux perturbateurs endocriniens, et à l'émergence de nouveaux qui peuvent poser un danger pour la santé. Comme mentionné auparavant, les perturbateurs endocriniens sont des imitateurs des hormones, mais le fait qu'ils ont plusieurs mécanismes d'action qui peuvent s'activer en parallèle dans plusieurs systèmes les rend difficiles à étudier. Un autre facteur de risque lié aux perturbateurs endocriniens et leur capacité de bioaccumulation, c'est-à-dire que leurs effets ne sont pas nécessairement apparents immédiatement, mais peuvent s'exprimer après plusieurs générations. Cela pose un vrai défi sur l'identification des sources de contaminants. Une catégorie importante de perturbateurs endocriniens émergents est les nanoparticules.

Les nanoparticules ont gagné en popularité avec le développement de la nanotechnologie et la nanomédecine. Elles sont aussi utilisées dans les produits plastiques et sont donc omniprésentes dans l'environnement. Les nanoparticules sont encore peu comprises concernant leur potentiel de perturbateurs endocriniens et posent un potentiel risque à cause de la bioaccumulation est c'est pour ces raisons et celles mentionnées auparavant que nous avons choisi de nous concentrer sur eux dans la seconde partie de ce projet.

b. Nanoparticules

Les nanoparticules sont une catégorie des nanomatériaux qui comprend des matériaux de taille entre 1 nm à 100 nm. Les nanoparticules sont aussi appelées des nanomatériaux de zéro dimension, car ils sont dans l'échelle nano en trois dimensions contrairement aux nanofibres par exemple qui elles sont en deux dimensions seulement. Les nanoparticules et nanomatériaux sont répandus dans de nombreuses industries de la production alimentaire aux applications

médicales. Malheureusement, tous les nanomatériaux ne sont pas sans danger pour la santé et beaucoup ne sont pas biocompatibles comme les nanocarbones qui sont utilisés en médecine. Plus spécifiquement les nanoparticules peuvent être divisés en trois catégories : les nanoparticules organiques ; les nanoparticules à base de carbone ; et les nanoparticules inorganiques.

Les nanoparticules organiques sont composées des composés biologiques comme des lipides, des glucides et sont typiquement biocompatibles et de faible toxicité. Elles sont utilisées en médecine dans des systèmes de délivrance de médicaments. Les nanoparticules à base de carbone sont faites exclusivement à partir d'atomes de carbone faiblement toxique. Elles possèdent, par exemple, des propriétés conductrices uniques, une résistance élevée, ainsi que des propriétés d'affinité électronique, optiques, thermiques et d'absorption. Elles sont utilisées dans les technologies d'administration de médicaments ainsi que dans des applications industrielles telles que le stockage d'énergie, la bio-imagerie, les dispositifs photovoltaïques, les équipements sensoriels, etc. Les nanoparticules inorganiques sont constituées de matériaux inorganiques comme les métaux, les céramiques et les semi-conducteurs (comme les oxydes métalliques). Les nanoparticules semi-conductrices sont utilisées dans la photocatalyse et dans les appareils électroniques pour leurs propriétés électriques uniques. Il est important de noter que leur profil toxicologique est peu connu comparativement aux deux autres catégories de nanoparticules. Ces nanoparticules ont habituellement une charge électrique qui les aide à prévenir leur agrégation, mais à cause de cette caractéristique elles sont très sensibles à leur environnement. C'est pourquoi nous nous sommes intéressés à trois nanoparticules semi-conductrices dans la présente étude : le titanium dioxyde (TiO_2), le zinc oxyde (ZnO), et le cérium dioxyde (CeO_2).

Le TiO_2 est utilisé dans différents types de capteurs de gaz composés d'oxygène, tels que les capteurs de monoxyde de carbone, les capteurs d'oxygène, les détecteurs d'ozone, et même les capteurs de méthane, ainsi que dans l'industrie alimentaire, notamment dans les aliments et emballages alimentaires. Elles sont également utilisées dans les contenants de produits personnels et dans la fabrication de verre autonettoyant, grâce à ses propriétés photo catalytiques. Malgré son utilisation répandue, l'autorité européenne de sécurité des aliments (AESA) ne considère pas le TiO_2 comme un additif alimentaire « sûr » parce qu'ils n'ont pas été en mesure d'établir une dose journalière sans risque. Les recherches sur son potentiel en tant que perturbateur endocrinien sont relativement limitées et ont été menées principalement sur le poisson-zèbre, où il a été démontré que l'exposition au TiO_2 entraînait une perturbation de la

glande thyroïde. Malgré le manque de données de ces effets sur l'humain, il existe des preuves que cette nanoparticule peut affecter certains animaux, et les effets dus à la bioaccumulation et aux effets transgénérationnels ne peuvent donc pas être écartés.

Le ZnO a des propriétés similaires au TiO_2, mais son usage est plutôt lié aux emballages, les contenants de produits personnels et les crèmes solaires en raison de leurs propriétés de protection contre les ultraviolets (UV) et la photocatalyse qui aident à prévenir le vieillissement des produits alimentaires et a des propriétés photo-protectrices dans les crèmes solaires. Comme le TiO_2, l'AESA n'a pas été capable d'établir une dose journalière sans risque pour la consommation humaine pour le ZnO. Comme un perturbateur endocrinien, le ZnO semble affecter principalement la vie aquatique en réduisant la fertilité. Bien que les effets sur l'humain puissent ne pas être immédiatement apparents, les effets sur des espèces animales, la bioaccumulation et des effets transgénérationnels décrits font du ZnO un candidat potentiel de perturbateur endocrinien pour l'humain.

Le CeO_2 est utilisé dans les capteurs d'oxygène, mais sa fonction première est la catalyse du monoxyde de carbone, comme ceux utilisés dans les masques à gaz, les convertisseurs catalytiques des voitures et les piles à combustible. Bien que ses applications ne soient pas directement liées à l'objet de notre étude, le CeO_2 est omniprésent et, en raison de sa similarité avec le TiO_2 et le ZnO, il constitue un bon troisième potentiel perturbateur endocrinien à étudier. En ce qui concerne son potentiel en tant que perturbateur endocrinien, il a été démontré chez la souris que le CeO_2 inhibait l'activité des enzymes antioxydantes et altérait la fertilité en diminuant la motilité et le nombre de spermatozoïdes, en augmentant les anomalies des spermatozoïdes et en provoquant des perturbations de l'équilibre endocrinien.

c. Le placenta humain

Le placenta est un organe transitoire, mais absolument essentiel pour que la grossesse et le développement de la progéniture. Le placenta n'est pas exclusif aux mammifères, il existe aussi chez les poissons non cartilagineux, certains amphibiens vivipares et les reptiles, mais sous une forme plus primitive. Le placenta joue un rôle dans les échanges gazeux, l'apport en nutriments et l'élimination des déchets, jouant ainsi le rôle des poumons, des reins et du foie du fœtus. Il agit également comme une barrière immunologique protégeant le fœtus de pathogènes susceptibles. Le placenta est un organe endocrine très important pour le maintien de la grossesse. Il maintient le corps jaune durant le premier trimestre, produit les hormones nécessaires à l'adaptation de la

physiologie maternelle et au développement du fœtus, et protège l'enfant des réactions immunitaires de la mère contre lui. Aussi, le placenta est responsable pour préparer la mère pour l'accouchement. Si le placenta est endommagé, cela pourrait affecter le développement de la progéniture ou même causer la mort de l'enfant et/ou de la mère.

Le placenta se développe à partir des cellules trophoblastiques du blastocyste. Au sixième jour, les cellules trophoblastiques s'attachent à l'utérus et commencent à l'envahir pour former ce qui deviendra le placenta (figure 1.3 a). Après l'implantation et l'invasion initiales, le trophoblaste se divise en deux : le trophoblaste cellulaire qui enveloppe l'embryon en développement et le trophoblaste syncytial qui continue à envahir l'endomètre de l'utérus. Le trophoblaste commence également à produire l'hormone chorionique gonadotrophine (hCG) pour maintenir la grossesse. Après la gastrulation, des cellules embryonnaires mésodermiques quittent l'embryon et commencent à s'accoler. sur le trophoblaste. À ce stade, la structure globale du trophoblaste et du mésoderme extrae-mbryonnaire s'appelle le chorion (figure 1.3 b). À ce stade, le chorion (contenant l'embryon) est entièrement contenu dans la paroi utérine, et le trophoblaste syncytial commence à s'ancrer dans l'endomètre maternel pour former des structures, appelées villosités choriales, autour desquelles le sang maternel s'accumule dans l'espace intervilleux. Le mésoderme remplit les villosités du côté embryonnaire, et donnera naissance aux vaisseaux sanguins du placenta (figure 1.3 b). À la 16e semaine, la placentation est terminée et les principales structures sont entièrement formées. Au fur et à mesure que le fœtus grandit, la partie du chorion qui fait face à la cavité utérine se rétrécit progressivement (figure 1.4 a). Le placenta mature se compose de la plaque choriale du côté fœtal, de la plaque basale du côté maternel et, entre les deux, de l'espace intervilleux. Les vaisseaux sanguins chorioniques du côté fœtal s'ancrent sur le côté maternel à travers l'espace intervilleux, où les vaisseaux sanguins maternels apportent des nutriments et du sang oxygéné au fœtus et éliminent les déchets et le sang désoxygéné. Dans le cas du placenta humain de type hémochorial, le sang maternel et le sang fœtal ne se mélangent jamais (figure 1.4 b).

Comme indiqué ci-dessus, le placenta joue un rôle endocrinien important, et cela le rend vulnérable aux effets des perturbateurs endocriniens, aggravé par le fait que le développement embryonnaire et fœtal est une période dynamique influencée par beaucoup de processus endocriniens. Les effets des perturbateurs endocriniens peuvent être multiformes, mais à ce jour les mécanismes et leurs effets sur le placenta et la progéniture ne sont pas bien connus. Il y a des perturbateurs bien connus dont nous connaissons leurs effets néfastes sur le placenta et le développement, ceux-ci sont les métaux lourds comme le cadmium (Cd) et le plomb (Pb) ; les

plastifiants comme les phtalates et leurs variantes, les xénœstrogènes comme le bisphénol A (BPA), et les filtres UV comme les parabènes. Le risque posé par ces perturbateurs endocriniens ne provient pas seulement de leurs effets biochimiques, mais aussi de leur ubiquité.

Le Cd est un composant courant des cigarettes. Il a été démontré que le Cd peut entraîner une diminution du poids et de l'efficacité du placenta, ce qui peut conduire à un retard de croissance du fœtus et à d'autres pathologies, dont beaucoup touche le système nerveux central. Le Pb est un composé qui traverse la barrière placentaire-fœtale. Le Pb peut augmenter l'apoptose chez le fœtus et interférer avec le réticulum endoplasmique et la fonction mitochondriale. En plus, le Pb entraîne une réduction de l'absorption de Ca^{2+} par le syncytiotrophoblaste, un processus important pour la fonction placentaire et la différenciation du trophoblaste. Le Pb perturbe également la production de sérotonine par le placenta, qui est importante pour la fonction placentaire et le développement du fœtus.

Les plastifiants tels que les phtalates et le BPA sont des xénœstrogènes connus. Il a été démontré que les phtalates et le BPA inhibent la différenciation des cellules trophoblastiques placentaires, et en particulier la capacité invasive du trophoblaste extravilleux et la différenciation du trophoblaste villeux, ce qui altère la placentation en général, perturbent la vascularisation et entraîne une restriction de la croissance et des anomalies épigénétiques dans le placenta. Enfin, les parabènes sont capables de traverser la barrière fœto-placentaire et de s'accumuler dans le fœtus. Ils induisent l'apoptose dans un modèle de cellules trophoblastiques placentaires et réduisent leur prolifération, ce qui perturbe la placentation et peut conduire à une grande variété d'anomalies du développement, voire à des fausses couches.

Les nanoparticules métalliques sont une autre classe de molécules ayant un fort protentiel de perturbation endocrinienne que l'on retrouve de manière omniprésente dans l'environnement moderne, mais qui sont encore peu connues. Les nanoparticules constituent une préoccupation croissante en matière de sécurité publique en raison de l'augmentation rapide de leur utilisation dans la vie quotidienne et dans diverses industries. Dans le contexte de la reproduction et du développement placentaire et fœtal, les nanoparticules ont un potentiel de traverser la barrière materno-fœtale en raison de leur petite taille. Il a été démontré que des nanoparticules d'un diamètre de 500 nm peuvent traverser la barrière placentaire et que l'exposition à des nanoparticules de 20 nm (200 ug/ml) et de 40 nm (500 ug/ml) induit l'apoptose dans les cellules de trophoblaste chez les souris. Dans le contexte du TiO_2, du ZnO et du CeO_2 plusieurs études réalisées sur des souris et des rats ont montré qu'ils ont un impact direct sur le développement du fœtus et sur la physiologie de la mère. L'exposition au TiO_2 a montré une augmentation de la

teneur en titane et une réduction du zinc et du calcium dans le sérum maternel, le placenta, ainsi qu'un gain de poids maternel, une réduction du poids fœtal et une diminution du nombre de fœtus vivants, avec un effet significatif sur le développement du squelette fœtal. Ces données indiquent que le TiO_2 peut atteindre et traverser le placenta. L'exposition au ZnO a une réduction du poids corporel des mères, une réduction du poids fœtal et une augmentation de l'incidence des anomalies morphologiques chez les fœtus. Enfin, l'exposition au CeO_2 a entraîné des dépôts de nanoparticules dans les cellules trophoblastiques placentaires. Il a également été démontré que cette nanoparticule peut réguler l'autophagie et diminuer la migration et l'invasion des lignées trophoblastiques HTR8/SVneo in vitro.

II. Problématique

La pandémie de la COVID-19 a entraîné une augmentation de l'utilisation des produits d'hygiène et de nettoyage, ainsi que des plastiques à usage unique. Cette augmentation soudaine et sans précédent peut entraîner des conséquences sur la santé humaine, dont les personnes en situation de vulnérabilité comme les personnes enceintes et leur fœtus. Ces conséquences sont, en partie, dues à des perturbations endocriniennes. Un des perturbateurs endocriniens potentiels est constitué par les nanoparticules d'oxyde métallique (TiO_2, ZnO, CeO_2) dont les effets endocriniens sur l'humain sont relativement mal connus, en particulier dans le contexte de la grossesse et plus particulièrement sur les fonctions et le développement du placenta.

III. Hypothèses et objectifs

1- La première hypothèse de ce projet de maitrise propose une augmentation de l'exposition environnementale aux contaminants liés aux produits de nettoyage, les plastiques et les cosmétiques dans la population générale en raison d'une utilisation accrue pendant la pandémie de COVID19. Pour vérifier ce postulat, le premier objectif est d'analyser les données relatives aux eaux usées municipales avant et après traitement afin de vérifier le niveau de contamination avant, pendant et après les confinements sanitaires pendant la pandémie.

2- La deuxième hypothèse est que les nanoparticules TiO_2, le ZnO et le CeO_2 présents dans les produits cosmétiques et alimentaires agissent comme perturbateurs endocriniens sur les cellules trophoblastiques. Pour vérifier ceci, le deuxième objectif de ce projet est

d’évaluer les effets sur la prolifération, la viabilité et l’apoptose de ces trois nanoparticules sur le modèle de choriocarcinome placentaire humain, les cellules JEG-3.

IV. Méthodologie

Partie 1 – analyse des eaux usées.

Nous avons analysé les données sur les eaux usées du Portail eau Québec du ministère de l'Environnement et des changements climatiques du Québec. Nous avons analysé des données de cinq stations d'épuration dans l'agglomération montréalaise pour les années 2017 à 2021. Les détails concernant chaque station se trouvent à la section 3.1. Les analyses statistiques ont consisté à calculer la moyenne des valeurs de contamination toutes les deux semaines. Nous avons compilé et traité les données à l'aide des logiciels R et GraphPad, en nous concentrant sur trois critères d’évaluation de la qualité de l'eau : i) la demande chimique et biochimique en oxygène à 5 jours partie carbonée (quantité consommée pendant 5 jours); ii) la demande carbonée; et iii) les matières en suspension.

Partie 2 - effets des nanoparticules sur les cellules trophoblastiques.

La lignée cellulaire de choriocarcinome placentaire JEG-3, un modèle de trophoblaste extravilleux humain, a été utilisée. Les cellules JEG-3 ont été cultivées dans un milieu EMEM 1X supplémenté avec des sels d'Earle, de la L-glutamine, 10% FBS, 100 mM de pyruvate de sodium et ≥99,5 % de tampon HEPES (acide 4-(2-hydroxyéthyl) -1-pipérazineéthanesulfonique). Les cellules sont incubées à 37^{o}C et 5% de CO_2.

Les nanoparticules étudiées sont le dioxyde de titane (TiO_2), l'oxyde de zinc (ZnO) et le dioxyde de cérium (CeO_2). Nous avons caractérisé les nanoparticules en termes de taille et de charge en utilisant respectivement la diffusion dynamique de la lumière et le test du potentiel zêta, avec les détails spécifiés à la section 3.3. Nous avons ensuite testé la viabilité des cellules JEG-3 à l'aide du test de viabilité MTT sur une période d'exposition de 24-h et 48-h à une concentration de 0 à 1000 mg/ml par intervalles 100 mg/ml de pour déterminer la valeur d’IC50 pour chaque nanoparticule (voir section 3.4 pour plus de détails).

En utilisant la valeur d’IC50 déterminée par MTT pour chaque nanoparticule, nous avons réalisé une analyse de cytométrie en flux pour déterminer la viabilité des cellules ainsi que leur état

apoptotique après le traitement. L'exposition a été réalisée pendant 48-h et la staurosporine a été utilisée comme témoin positif pour l'apoptose (voir la section 3.5 pour plus de détails).

Ensuite, nous avons réalisé des analyses d'immunocytochimie pour visualiser les noyaux des cellules dans les cellules exposées 48-h au IC50 pour chaque nanoparticule. Les mêmes réplicas que pour les analyses de cytométrie en flux ont été utilisés. Les immunocytochimies ont été visualisées à l'aide d'un laser d'une longueur d'onde de 330 - 380 nm avec un grossissement de 200 X. Les détails de la méthode sont présentés à la section 3.6.

Nous avons réalisé 3 réplicas techniques et 3 réplicas biologiques pour les essais MTT. Les valeurs aberrantes ont été déterminées à l'aide d'un test de Grubb sur GraphPad avec une valeur alpha de 0,2. Les données de viabilité obtenues par cytométrie en flux sont une moyenne de 3 répliqua biologiques (section 3.7).

V. Résumé des résultats

Partie 1 – analyse des eaux usées.

Les résultats des données sur les eaux usées ont confirmé ce qui avait été rapporté dans d'autres études dans le monde : le premier confinement sanitaire due à la pandémie a conduit à une amélioration globale de la qualité de l'eau, au moins temporairement, mais dans la plupart des cas, les niveaux de contamination sont revenus à la normale ou ont augmenté par la suite (figure 4.1). Nous avons également observé une augmentation de la moyenne annuelle de la demande chimique en oxygène en 2020 et à nouveau en 2021, ainsi qu'une augmentation progressive de 2017 à 2021 pour la demande biochimique en oxygène à 5 jours partit carbonée (figure 4.2). Nous avons observé une réduction constante de la qualité des eaux usées à l'île Notre-Dame pour les trois catégories de contamination du début de la pandémie jusqu'en 2021 (figure 4.3), ce qui suggère que les confinements ont entraîné une réduction de la contamination de l'environnement, en particulier dans les centres d'attraction tels que l'île Notre-Dame.

Curieusement, nous avons observé une diminution de la qualité des eaux usées provenant du nord du Québec à partir de l'été 2021 (figure 4.4), ainsi qu'une diminution de la moyenne annuelle de la qualité de l'eau pour 2021 dans les trois catégories analysées (figure 4.5). Ceci suggère que la reprise des activités humaines avec la levée totale de la plupart des restrictions sanitaires a conduit à une augmentation des activités et donc augmentations des rejets dans les eaux usées.

Partie 2 - effets des nanoparticules sur les cellules trophoblastiques.

Les trois nanoparticules étudiées se sont révélées sans charge dans le milieu de culture cellulaire avec des tailles d'agrégats de 422,7 nm pour le TiO_2, 12,93 nm et 61,92 nm pour le ZnO, 13,66 nm et 89,05 nm pour le CeO_2.

Les essais de viabilité cellulaire ont montré que les cellules JEG-3 sont résistantes aux nanoparticules jusqu'à une certaine concentration, après quoi leur viabilité commence à diminuer rapidement. L'effet le plus prononcé et le plus constant a été obtenu avec le ZnO, les résultats étant similaires après 24-h et 48-h de traitement. Les valeurs d'IC50 pour le TiO_2, le ZnO et le CeO_2 sont respectivement de 963,4 µg/ml, 153,2 µg/ml et 903,2 µg/ml à 24 heures de traitement et de 836,3 µg/ml, 146,5 µg/ml et 772,2 µg/ml à 48 heures de traitement. (Figure 4.6)

Les analyses de cytométrie en flux ont démontré que les nanoparticules tendent à induire l'apoptose des cellules JEG-3 (figure 4.7), mais seul le ZnO a montré une réduction significative de la viabilité par rapport aux cellules non traitées (figure 4.8). Ces résultats apparemment contradictoires nous ont conduits à réaliser des analyses d'immunocytochimies pour déterminer l'effet des nanoparticules sur les cellules individuellement. Nous avons observé une réduction globale des noyaux après l'exposition aux trois nanoparticules, ainsi qu'une augmentation de la proportion de noyaux fragmentés, ce qui indique une augmentation de la mort cellulaire et de l'apoptose.

VI. Conclusion

Notre étude montre que si la pandémie de covid-19 a eu des effets positifs tels que la réduction temporaire de la contamination de l'eau au cours de la phase initiale de confinement, il semble que le changement des habitudes de vie, ainsi que les mesures sanitaires mis en place qui ont encouragé l'utilisation de plastiques à usage unique et l'utilisation de produits nettoyants et de décontaminant ont conduit à une augmentation globale de l'exposition observée par l'augmentation de la contamination des eaux usées en espèces oxydantes, ce qui pourrait conduire à un nouvel ensemble ou à une incidence accrue de pathologies connexes.

Au vu de nos données concernant les effets des nanoparticules, celles-ci suggèrent que le TiO_2, le ZnO et le CeO_2 ont un effet néfaste sur la viabilité des cellules placentaires JEG-3. En effets, elles induisent l'apoptose à des degrés divers, le ZnO ayant les effets les plus importants. Bien

qu'il soit intéressant de noter qu'il y avait un certain degré de divergence dans nos résultats, en particulier entre l'essai de viabilité et les résultats de la cytométrie en flux, les données de microscopie ainsi que la littérature soutiennent notre hypothèse selon laquelle les trois nanoparticules auraient un effet néfaste sur la viabilité et induiraient l'apoptose des cellules trophoblastiques.

Dans le contexte d'une exposition accrue à des perturbateurs endocriniens les nanoparticules métalliques sont susceptibles d'avoir un effet néfaste sur les fonctions et la formation du placenta, qui à son tour peut avoir des effets délétères sur le développement embryonnaire et fœtal, ce qui pourrait avoir un impact durable sur la qualité de vie d'un grand nombre d'enfants et de personnes. D'autres études devront être menées sur les effets possibles des nanoparticules sur la viabilité, telle que l'augmentation des espèces oxydatives réactives, les effets sur d'autres structures cellulaires telles que le cytosquelette, la membrane cellulaire et les mitochondries, et idéalement, répéter l'expérience sur la culture de cellules trophoblastes primaires et d'explants placentaires afin de confirmer notre hypothèse.

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LIST OF ABBREVIATIONS

BPA – Bisphenol A

WQI – Water Quality Index

COVID-19 – Corona Virus Disease 2019

SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2

QAC - Quaternary ammonium compound

EDC – Endocrine disrupting chemical

TiO_2 – Titanium dioxide

ZnO – Zinc oxide

SiO_2 – Silicon dioxide

CeO_2 – Cerium dioxide

Wnt - Wingless

hCG - Human chorionic gonadotropin

hPL - Human placental lactogen

LH - Luteinizing hormone

CTB - Cytotrophoblast

EVCT – Extravillious cytotrophoblast

VT – Villous trophoblast

ST - Syncytiotrophoblast

Cd - Cadmium

Pb – Lead

BaP - Benzo(a)pyrene

PCE - Portail des connaissances sur l'eau

MES – Matière en Suspension

DCO - Demande chimique en oxygène

DBO5C - Demande Biochimique en Oxygène 5 jours, Partie Carbonée

TTS – Total suspended solids (suspended materials)

COD – Chemical Oxygen Demand

BOD5C - Biochemical oxygen demand over 5 days carbonaceous demand

INSPQ - Institut national de santé publique du Québec

FBS – Fetal bovine serum

PBS - Phosphate buffered saline

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

ICC - Immunocytochemistry

AIF - Apoptosis-inducing factor

NADH - Nicotinamide adenine dinucleotide hydrogen

FAD - Flavin adenine dinucleotide

DoHad - Developmental origin of health and diseases

1 Introduction

1.1 The COVID-19 Pandemic

1.1.1 Origins of the COVID-19 Pandemic

The Coronavirus 2019 (COVID-19) pandemic emerged in late 2019 and quickly spread across the globe in a matter of months, being declared a worldwide pandemic on March 11, 2020, by the World Health Organization (WHO) (CDC, 2023). COVID-19 is caused by the SARS-CoV-2 virus, which is closely related to the renamed SARS-CoV-1 that caused the disease, commonly known as SARS, in the early 2000s, and like SARS, COVID-19 has its origins in the wet markets of China (Keshta, et al 2021). COVID-19 emerged in Wuhan, China from the Huanan seafood wholesale market, via zoonotic transmission of the virus due to the consumption of infected animals, most likely bats, which are known as carriers of coronaviruses. (Keni, et al. 2020). COVID-19 presents as a relatively mild, although highly contagious respiratory illness with an incubation period of up to 14 days (Hu, et al. 2020). In Canada, the virus primarily spread from the United States and the Middle East with only 7.6% of cases originating from China, out of a total of 118 initial COVID-19 cases tracked by March 11, 2020 (Zhao, et al. 2020). The very first case in Canada was recorded on January 25, 2020, and on March 13th public states of emergency have been declared successively in all provinces and territories starting with Quebec on the same day, ending with Yukon on March 27th (Urrutia, et al. 2021).

1.1.2 Timeline of COVID-19 pandemic in the Montréal metropolitan area

To illustrate when the COVID-19 pandemic had the biggest impact on daily lives in the region of Montréal, a timeline of the pandemic in 2020 and 2021 showing major events related to public health and regulations during these two years was built. We further looked at the INSPQ to confirm compare our timeline with the official dates of the different COVID-19 waves (table 1).

As shown in table 1, the pandemic effectively started on March 13, 2020, with the initial cancellation of public events, and complete closure of all non-essential businesses on the March 24, until May 27. This represents the first major wave of COVID-19. The partial reopening of public spaces was maintained until October 1, 2020, when the government of Québec had imposed a lockdown due to the 2nd major wave of SARS-CoV-2 infection. This extended into 2021 where quarantine was imposed with some restrictions lifted in February, a premature measure, which leads to the extension of the lockdown into the end of May 2021. The vaccination campaign which began in March 2021 in Quebec led to a gradual reopening of public spaces starting on May 25,

2021. This continued until the appearance of the Omicron variant at the end of 2021 where another lockdown has been imposed in the province of Quebec.

Table 1.1 **Timeline of the COVID-19 pandemic in the Quebec**

Year 2020	Event
13/03	Initial cancellation of public events and facilities
15/03	Closure of reactional venues, restaurants half capacity
20/03	Indoor gathering prohibited; social distance outdoors mandatory
24/03	Closure of all non-essential businesses
28/03	Interprovincial travel restricted
31/03	All essential business close on Sundays
01/04	Interprovincial travel is restricted even further
04/05	Retail stores reopen outside the Montréal region, travel restrictions lifted
11/05	Pre-secondary Schools outside of metropolitan Montréal reopen, retail stores in Montréal can reopen
22/05	Outdoor gatherings of up to 10 people allowed
27/05	Reopening of non-essential self care facilities, museums, libraries, camping sites outside of Montréal region
30/05	Suspension of pool activities lifted
18/07	Mandatory indoor masks
01/10	Province put under red alert due to rise of COVID-19 infection cases (restriction on indoor gatherings and dine in businesses
05/10	Closing of gyms, team sports, masks required in schools
25/12	Closure of non-essential stores

Year 2021	Event
06/01	Lockdown extended for 4 weeks with quarantine measures put in place
02/02	Some restrictions lifted such as on nonessential stores, museums, and higher education institutions can gradually move to in person classes
16/02	Further restrictions were lifted in preparation for spring break
01/03	Vaccination of 80 and older starts
10/03	Vaccination of 70 and older starts
12/03	Vaccination of 65 and older starts
17-26/03	Curfew pushed to 9:30p.m., sec 3-5 go back to in person classes, gyms, show venues reopen. Less affected areas of Quebec have curfew abolished

Year 2021	Event
29/03	State of emergency extended
05/04	Lockdown extended to May 4
30/04	Vaccination starts for 50 – 59-year-olds
04/05	Lockdown extended to May 11
14/05	Vaccination becomes available to all adults
25/05	Nonessential businesses in greater Montréal reopened
28/05	End of Curfew and further reopening with limited occupancy
30/05	Montréal reopens public pools, etc.
07/06	Montréal and Laval move to the orange zone (gyms, indoor dining reopening etc.).
14/06	All orange zones move to yellow zones
28/06	Entire province moves to the green zone
01/08	Further reduction of restrictions in green zones namely in how many allowed to gather in indoor locations
23/08	Montréal ends state of emergency
01/09	Vaccine passport takes effect for virtually all non-essential activities
Aug-Sept	Schools and higher education return to full in person learning
02/11	Remote work no longer recommended
15/11	Further reduction in restrictions in bars and restaurants (no need to keep client list)
29/11	First two cases of omicron in Canada revealed to have passed through the Montréal airport (YUL)
20/12	Due to the Omicron variant the province shuts down again with exception to restaurants with reduced operating hours. Plans to phase out remote work are thwarted, and schools are suspended until 10/01/2022
30/12	New Curfew is instated from 10 p.m. to 5 a.m. complete shutdown of the province, and private gathering prohibition

Green text – loosening of restrictions, red text – imposition of restrictions.

1.1.3 COVID-19: usage of cleaning, and sanitation products, and waste generation

The COVID-19 pandemic led to a drastic change in human habits, one of the most major ones being our habits with regards to the usage of cleaning and sanitation products both in terms of frequency and amount. Several surveys show that as many as 92.7% of respondents increased their use of hand sanitizers, 87.4% increased their use of soap and roughly 70% increased their use of surface disinfectants as well bleach (Vayisoglu, et al. 2021; Gharpure, et al. 2020). If

misused these products have the capacity to cause serious harm, primarily as a skin irritant, but even worse, poisonous effects that occur due to inhalation and sometimes even dermal exposure. Alcohols alone can lead to skin irritation and poisoning even through skin exposure, especially in children, with much more severe effects due to accidental ingestion. Even spills of relatively mild products such as isopropyl alcohol, which are made to be applied directly on the human body, can contaminate ground water, meaning products such as surface cleaners are likely far more dangerous both due to direct exposure and if their seep into the environment (Vayisoglu. S.K., et al. 2021; Mahmood. A, et al. 2020). In addition to cleaning and sanitation products, the pandemic was associated with an increase in use of plastics across all sectors as well as the emergence of a new behavior, the regular usage of medical protective equipment among the general population, namely gloves and masks. Plastics and gloves are known to contain materials which have the potential to act as both environmental pollutants and direct toxins to humans, such as the presence of microplastics, and chemicals such as Bisphenol A (BPA) which have long been studies for their toxicological effect when they shed into the environment from plastic waste (Kadac-Czapska, et al. 2023; Teuten, et al. 2009). The increase in use lead to an increase in direct exposure to contaminants, but perhaps more important to the increased shedding of contaminants into the environment through material degradation which can affect human health in the long term via bioaccumulation making the effects of these contaminants not be readily apparent which could possibly affect the human population and animal population years down the line (Jedruchniewicz, et al 2021). An alarming example was described from the medical field throughout COVID-19 detection tests. Indeed, it was shown that PCR COVID-19 test generated over 15 thousand tons in plastic residue waste by August 2020 (Mittal, et al. 2022).

1.1.4 Impact of the COVID-19 pandemic on municipal wastewater

As discussed in the section 1.1.3, there are major environmental concerns and their impact on human health due to the shift in human habits during the COVID-19. Despite the risk posed by increased use of plastic and cleaning products, it was observed that due to the reduction of human activity during the COVID-19 pandemic, overall pollution has gone down. Both air and water quality have improved, which is associated with the measured related to the COVID-19 pandemic. For example, beaches were cleaner, and the restoration of some aquatic life due to the reduction in fishing was described and, a reduction in noise pollution (Loh, et al 2022). More specifically, concerning municipal wastewater, a case study in Malaysia that measured surface water quality on river water via existing water quality stations measured the Water Quality Index (WQI), which in Malaysia consists of 6 variables: dissolved oxygen, biochemical oxygen demand, chemical

oxygen demand, ammonia nitrogen, suspended solids, and pH (Chidiac, et al. 2023), with a lower value being better, and it is divided into 5 classes with class-I being the best water quality and Class-V being the worst (Table 2). It was shown that through January 2020, February 2020, and March 2020 (the month where most countries went into a general lockdown) the WQI measurements shifted from over 90% falling in Class-II in January, to approximately 75% in February, to 90% in Class-I in March, meaning that there was a drastic water quality increase at the beginning of the lockdown periods (Najah A., et al. 2021). Another similar study done on Bhutan-India-Bangladesh transboundary rivers, showed similar observations with WQI measurements during lockdowns, with the addition of post unlock measurements. It shows that on average there is some increase in WQI value to an intermediate value between pre-lockdown and lockdown values, although with a much tighter distribution than pre-lockdown values. Individually out of 10 stations, while some maintained post lockdown values after reopening, some showed values even higher than the pre-lockdown (Sarkar. S, et al. 2021). This indicates that while lockdowns and reduced human activity has a clear positive effect on water pollution, the effects after reopening can be unpredictable and need to be evaluated on a case-by-case basis.

Table 1.2: Water Quality Index values and interpretation

Water Quality Index (WQI)	Water Quality Status	Possible Uses
0–25	Excellent Water Quality	Drinking, Irrigation, And Industrial
26–50	Good Water Quality	Drinking, Irrigation, And Industrial
51–75	Poor Water Quality	Irrigation And Industrial
76–100	Very Poor Water Quality	Irrigation
>100	Unfit For Consumption	Proper Treatment Required

(Sarkar, et al. 2021) Red: Class-V, Orange: Class-IV, Yellow: Class-III, Green: Class-II, Blue: Class-I

Thus, the COVID-19 Pandemic had both positive and negative effect, but it is important to note that the positive effects such reduction in noise, air and water pollution are only temporary and specifically tied to COVID-19 pandemic measures and reduction in human activity. Meanwhile, the increase in exposure to harmful chemicals and plastic waste can have detrimental effects that could affect individuals even across generations due to bioaccumulation effects (Espejo, et al. 2020).

1.1.5 Emerging contaminants during the COVID-19 pandemic

The increase in exposure to cleaning and sanitizing products as well as plastic pollutants has the potential to introduce new contaminants or make the presence of existing contaminants much more and thus pose a significant risk to human health. In other words, the pandemic paved the way for the emerging contaminant to pose a new risk for human health. As mentioned in section 1.3, alcohol not made for human consumption, that's used for sanitizing purposes, can easily become toxic through excessive skin contact, inhalation, or accidental ingestion, with children being the most vulnerable population (Mahmood, et al. 2020). Moreover, plastics, surface cleaners and soaps pose their own risks for human health (Mittal, et al. 2022). Plastics pose a risk for the environment through their degradation which leads to shedding of microplastics and nanoplastics and the release of chemicals such as BPA. As discussed in section 1.3 they pose a danger both directly but especially due to bioaccumulation and potentially act as endocrine disruptors affecting both male and female reproductive systems, the adrenal, pituitary, and thyroid glands, and even the hypothalamus (Cai, et al. 2023; Lambert, et al. 2016; Ullah, et al. 2022). Specifically, BPA is a known xenoestrogen, or an estrogen mimicker, meaning it can bind to estrogen receptors and induce the pathways that endogenous estrogen would normally induce. A study in mice showed that BPA induces identical morphological alterations in mice uterus and vagina as when exposed to estradiol (Steinmetz, et al. 1998). In the case of surface disinfectants, the main products that can pose a risk to human health are quaternary ammonium compounds (QACs, or "quats). A study showed that collected dust samples prior to and during the pandemic showed a significant increase in QAC concentrations (median increase from 36.6 mg/g to 58.9 mg/g) with higher concentrations in households that reported disinfecting more often, indicating a correlation between frequency of usage and increased exposure (Zheng, et al. 2020).It was shown that QACs reduce fertility in both male and female mice by affecting sperm concentration and motility, and reduced the number of corpora lutea after 8 weeks of exposure respectively (Melin, et al. 2016), suggesting and endocrine disrupting effect. Soaps, shampoos, and many self-care products contain phthalates and, triclosan added as liquid plasticizers, and for antimicrobial activity respectively, both considered endocrine disruptors (Diamanti-Kandarakis, et al. 2009). A Swedish study showed a direct correlation between the increased usage and exposure to products containing these compounds and increased concentrations in urine samples in mothers and their children (Larsson, et al. 2014).

1.1.6 Endocrine-Disrupting Chemicals (EDCs)

Endocrine-disrupting chemicals are chemicals that can interfere with, mimic, or even block endogenous hormones, thus interfering with the endocrine system of the organism (Diamanti-Kandarakis, et al. 2009). As a result, there are many ways by which their effects can manifest and act throughout a multitude of mechanisms of action. EDCs can act as direct receptor agonists or antagonists, by up or down regulating receptor expression or signal transduction. They can also act on hormone synthesis, transport, breakdown/clearance, circulation, or distribution (Merril, et al. 2019). A single EDC could have multiple mechanisms of action which further complicates our ability to understand how they might affect human health both short and long term (Gingrich, et al. 2020).

An important class of emerging EDCs is nanoparticles. Nanoparticles themselves and their mechanism of action are relatively poorly understood compared to the other EDCs mentioned above since their usage and application in human life and different fields is relatively new and hasn't been studied as extensively. Their emergence is related to the field of nanotechnology and nanomedicine, but they are also used in common products such as food packaging, plastic containers, food items themselves, and can even interact with surface disinfectants leading to their increased aggregation and thus increased exposure. Thus, our exposure to nanomaterials is ubiquitous, and despite apparent safety, studies suggest that they can have endocrine disrupting effects (Gupta, et al. 2018; Onyeaka, et al. 2022, Radwan, et al. 2021). For these reasons we decided to focus our study on the impact of nanoparticles as potential endocrine disruptors due to their presence in single use plastics and potential interactions with cleaning products, both of which saw a rise in use during the pandemic, and the relative novelty compared to other more well-established compounds.

1.2 Nanoparticles as endocrine disruptors

1.2.1 Types and characteristics of nanoparticles

Nanoparticles are a subset of nanomaterials, which comprises materials at the nanoscale, typically between 1 nm to 100 nm in size, nanoparticles specifically are referred to as “zero dimensions” nanomaterials since they are at the nanoscale in all three physical dimensions, as opposed to nanofibers which have one dimension significantly larger (Joudeh, et al. 2022). As mentioned in section 1.1.6, nanomaterials and nanoparticles are a growing field in a variety of industries, from food production, such as nanoparticle additives that enhance food quality in some way, (ex.; addition of titanium dioxide (TiO_2) as a brightener, zinc oxide (ZnO) to packaging and silicon dioxide (SiO_2) as a thickening agent (Onyeaka, et al. 2022) and to medical and industrial applications, such as carbon nanomaterials (carbon nanotubes for example) which allowed for superior methods of bioimaging, biosensor and drug delivery due to their low toxicity and biocompatibility (Bayda, et al. 2019).

Nanoparticles can be classified into three main classes: organic nanoparticles, carbon-based nanoparticles, and inorganic nanoparticles (Joudeh, et al. 2022). Organic nanoparticles are comprised of what can be considered biological components, that are like those found in living organisms, such as proteins, carbohydrates, lipids, and different types of polymers. They are typically very biocompatible and of low toxicity, and as such are used in medical instruments and targeted drug delivery systems (Joudeh, et al. 2022). Carbon-based nanoparticles are made exclusively from carbon atoms and are also characterized by relatively low toxicity, and they possess properties as conductivity, high strength, electron affinity, optical, thermal, and sorption properties. As such they are used in drug delivery technologies and in industrial applications such as energy storage, bioimaging, photovoltaic devices, sensory equipment and more (Joudeh, et al. 2022). Inorganic nanoparticles are made of inorganic materials which can consist of metals, ceramic, and semiconductor (such as metallic oxides, nanoparticles of interest in the present master project). Semiconductor nanoparticles are used in photocatalysis as well as optic and electronic devices due to their unique electrical properties which allow for higher fine tuning of electrical conduction (Joudeh, et al. 2022). Most importantly, unlike the other two classes of nanoparticles, the toxicological profile of inorganic nanoparticles is far more variable and is rather poorly understood, in the present master project we will focus on TiO_2, ZnO, and Cerium oxide (CeO_2). As mentioned above, metallic oxide nanoparticles have semi-conductive properties and posses a charge which helps in preventing their aggregation, but their properties are highly influenced by their environment which gives them a wide array of applications, but more

specifically, TiO_2, ZnO are used in food preparation and packaging as well as sunscreens and CeO_2 is used for its catalytic properties against carbon monoxide (Joudeh, et al. 2022; Chavali, et al. 2019).

1.2.2 Titanium Dioxide (TiO_2)

TiO_2's applications range from gas sensors such as carbon monoxide sensors, oxygen sensors, ozone sensors, and methane sensors, to a wide range of applications in the food industry (ex: in food itself to whiten/brighten it, food packaging) and the containers of personal care products, in self-cleaning glass where its photocatalytic properties help protect against degradation due to UV radiation exposure which in the former, and the same UV interaction helps break down organic dirt in the latter (Chavali, et al. 2019; Onyeaka, et al. 2022; Zholobak, et al 2011; Foster, et al. 2011). Despite their rampant use in the food industry both as a food additive and a component of plastic packaging used to as transparent UV light absorbers, since 2020 the European Food Safety Authority (EFSA) no longer considers TiO_2 to be safe to be used as a food additive. This follows an investigation into its genotoxicity that found there is no way to establish safe daily intake levels which exacerbated by the fact that nanoparticles do not only pose an immediate risk as toxins but also bioaccumulate which can pose a risk even across generations (EFSA, et al. 2021). Research on its potential endocrine disrupting effect is relatively limited and was mainly studied on zebrafish where it was demonstrated that TiO_2 exposure led to enhanced thyroid endocrine disruption and impeded neuronal development (Lei, et al. 2020). Despite the lack of data on its effects on humans, there is evidence that it affects other animals such as zebrafish, suggesting that bioaccumulation and cross-generational effects cannot be discarded.

1.2.3 Zinc Oxide (ZnO)

ZnO shares many characteristics and applications in similar industries with TiO_2 such as in gas sensors, in food packaging, in the containers of personal products as well as in sunscreens due to their UV-shielding and photocatalytic properties (Chavali, et al. 2019; Onyeaka, et al. 2022; Zholobak, et al 2011). ZnO is also used in plastic packaging used during the COVID-19 pandemic as discussed previously. Like TiO_2, despite its ubiquitous usage, the EFSA revaluated its use in food materials (i.e., food packaging). They found that despite compliance with specific migration limit (number of nanoparticles that are transferred from the plastic packaging to the food) there is no way to ensure that the upper limit of ZnO consumption is exceeded due to exposure to other sources, and similarly to TiO_2 its risk comes primarily from bioaccumulation (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2016). ZnO had been mainly studied in aquatic life as an endocrine disruptor where it interferes with Wingless-related

integration site (Wnt) signalling pathways in hydra (Yamindago, et al. 2018) and lead to growth restriction and reduced reproductivity in daphnia (Qianju, et al. 2022). While there are no apparent effects on humans especially through skin exposure (such as through sunscreens), exposure through ingestion and inhalation is still a concern when it comes to sunscreen usage and as previously mentioned, plastic packaging usage (Ruszkiewicz, et al. 2017). Similarly, to TiO_2, even though the effects on humans might not be readily apparent, the effects on other species of animals such as hydra and daphnia and potential effects due to bioaccumulation cross-generationally cannot be discarded making ZnO a plausible endocrine disruptor in humans.

1.2.4 Cerium Dioxide (CeO_2)

Cerium Dioxide (CeO_2) is also used in oxygen sensors, but its primary function is in catalysis of carbon monoxide such as in gas masks, in catalytic converters of cars, and fuel cells (Chavali, et al. 2019; Dey, et al. 2020). CeO_2 is ubiquitous in modern society, and due to its similarity to TiO_2 and ZnO, is a good potential endocrine disruptor to study. It was shown in mice that CeO_2 inhibited the activity of antioxidant enzymes, decreased sperm motility and count, as well as increased sperm abnormalities, and lastly, elicited endocrine balance disruptions by reducing the production of LH and FSH, reduce haemoglobin levels, PCV, and RBC levels, and reducing testosterone (Adebayo, et al. 2018). So CeO_2 is already known to cause endocrine disruption in mammals, However, the long-term effects of CeO_2 exposure are unknown.

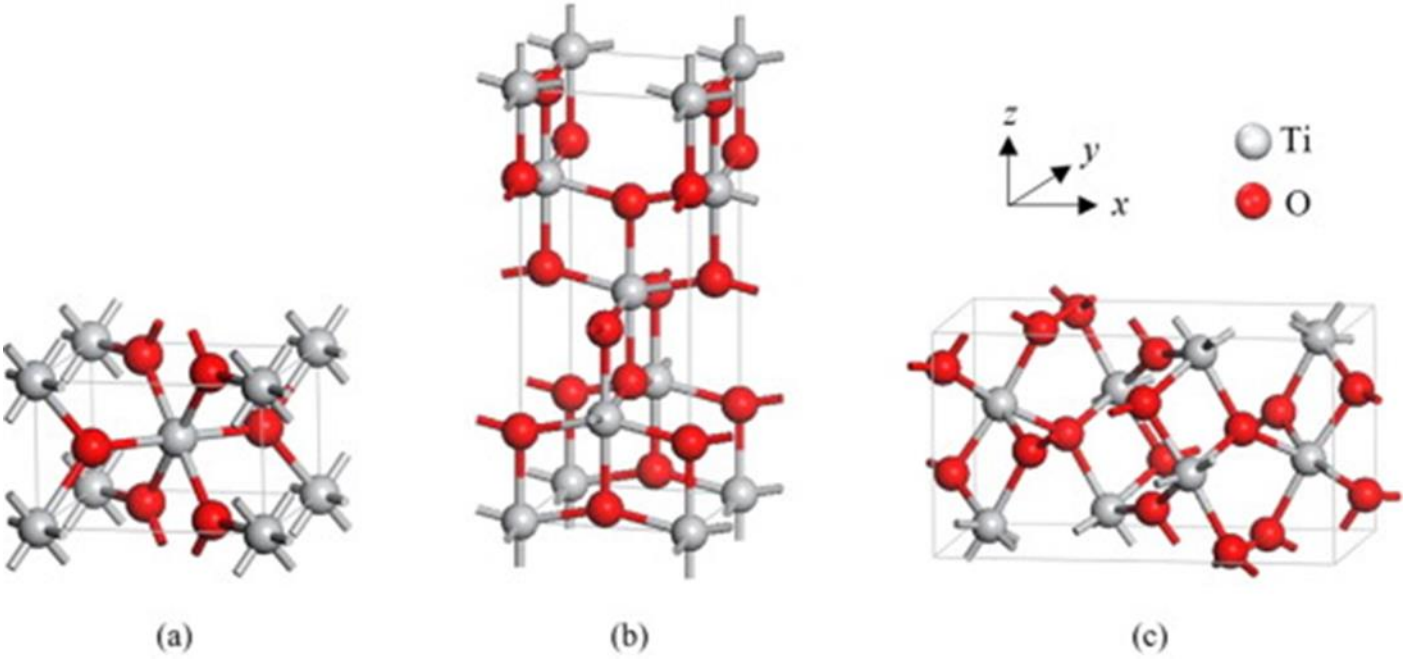

Figure 1.1: The three phases of the crystal structure of titanium dioxide (TiO_2) (a) rutile, (b) anatase, and (c) brookite (Gackowski, et al. 2023).

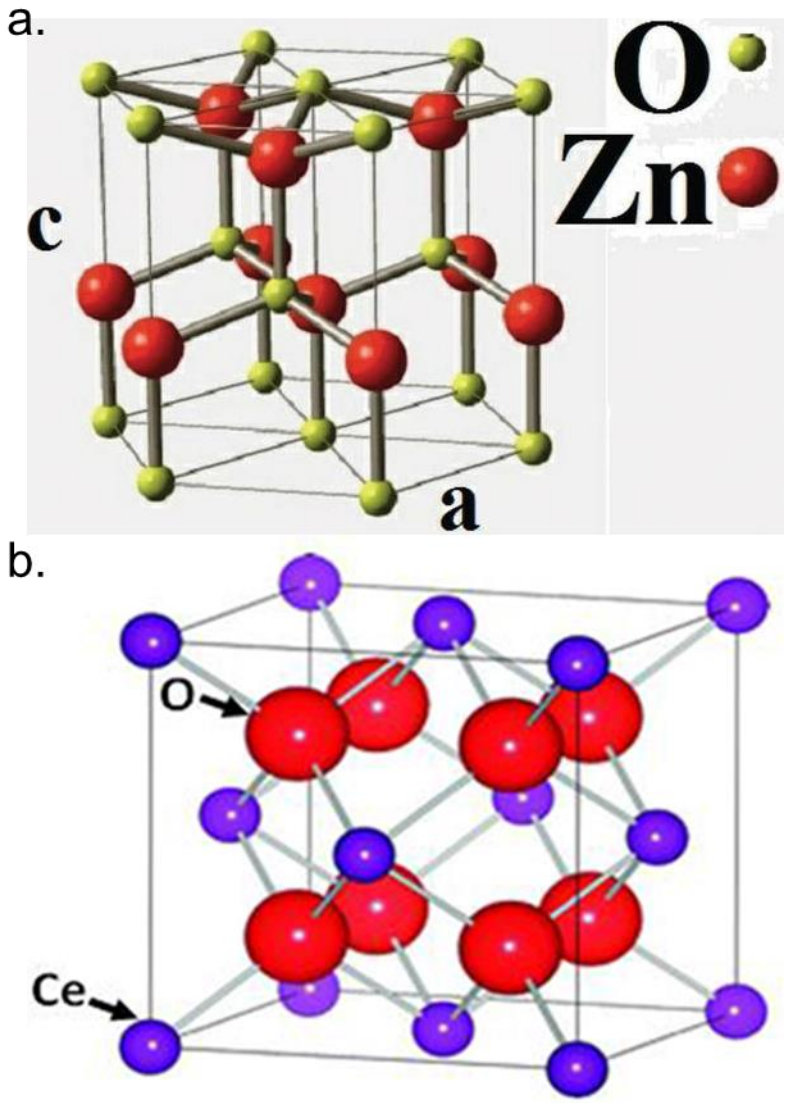

Figure 1.2: Crystal structure of (a) zinc oxide (ZnO), and (b) cerium dioxide (CeO_2) (Al Hardan, et al. 2017; Younis, et al. 2016).

1.3 The Human Placenta

1.3.1 The Role of the Placenta

The placenta is a key organ in human development that is often overlooked because it is only seemingly relevant in the relatively short period of pregnancy. This misconception led to it often being ignored by medical science (Badawi, et al. 2000). As a transitory organ, the role of the placenta and its convergent evolution allowed for immense diversification of animal life and ultimately for the evolution of mammals and humans. The organ is not exclusive to mammals but appeared in a more primitive form in non-cartilaginous fish, some viviparous amphibians, and reptiles, and later in mammals, meaning that understanding the function of the placenta has important implications not just for medicine but understanding life on earth itself (Roberts R Michael. 2016). The placenta plays a vital role in supporting the fetus and ensuring it thrives. Damage to or placental disorders can lead to reduced fetal growth, and even death. It plays a role in gas exchange, nutrient supply, and waste removal, effectively acting as the fetus's` lungs, kidneys, and liver. It also acts as an immunological barrier protecting the fetus from a host of pathogens that might affect the mother. At the same time, antibodies from the mother pass onto the fetus and provide short term immunity post partum (Gude, et al. 2004; Taylor, et al. 2017, Burton, et al. 2015, Redman. 1986). The placenta is also an endocrine organ, specifically secreting human chorionic gonadotropin (hCG), progesterone, estrogen and human placental lactogen (hPL). In the placenta, hCG is produced by villous syncytial trophoblast cells and hyperglycosylated hCG is produced by cytotrophoblast cells (Cole. 2010). Syncytial trophoblast hCG takes over the function of LH to maintain the progesterone production of the corpus luteum for approximately 4 weeks after implantation (Figure 1.3, a,b) after which there is enough syncytial trophoblasts to take over progesterone production (Cole. 2010). Hyperglycosylated hCG is an autocrine which act on cytotrophoblasts to promote their own cellular growth and invasion of the uterine wall (Cole. 2020). Progesterone maintains the pregnancy by upregulating and attenuating the vascular buildup and vascular proliferation in the uterus to make it suitable for a pregnancy, as well as downregulating hormones which are involved in the commencement of labor and post partum symptoms, such as the influx of oxytocin and prostaglandin, also reducing immune reactions against the fetus. Estrogen shares some of the former functions of progesterone but also promotes the physiological changes in the mother during pregnancy to accommodate the growing offspring as well as prepare the mother for birth. The hPL acts to reduce maternal insulin sensitivity during pregnancy which subsequently will also reduce its consumption of glucose to

ensure the sufficient supply of glucose to the offspring, as well as prepare the mother for lactation, and maintain lactation post partum to nourish the child (Napso, et al. 2018).

1.3.2 The structure of the placenta

The human placenta is a feto-maternal organ, but it is developed from the trophoblast cells of the blastocyst embryo. At day 6 the trophoblast cells attach themselves to the uterus and start invading it to start forming what will eventually become the placenta. Additionally, the embryo at this stage also consists of the inner cell mass which is what will eventually develop into the offspring (figure 1.4 a). As such, the placenta has the same genetic code (and thus sex) as the embryo (Burton, et al. 2015). Following initial implantation and invasion, the trophoblast divides into two: Cytotrophoblast which envelops the developing embryo. The syncytial trophoblast which further invades into the endometrium of the uterus and starts secreting hCG hormone to maintain the pregnancy. Once the formation of the embryonic disc and gastrulation start (which is the differentiation of mesodermal cells), some of these cells leave the embryo and start lining up the cytotrophoblast. At that point the overall structure of the syncytial trophoblast, cytotrophoblast and extraembryonic mesoderm is called the chorion and will be referred to as such (figure 1.5 b). At this point, the chorion (containing the embryo) is fully contained within the uterine wall, and the syncytial trophoblast start to anchor itself into the maternal endometrium and form almost root-like structures that are called the chorionic villi around which maternal blood pools in spaces called intervillous spaces. The mesoderm fills these villi from the embryonic side and will eventually give rise to the blood vessels of the placenta (figure 1.4 b). By week 16, the placentation is complete, and the main structures are fully formed. The only real change that will follow now is that as the fetus grows, the side of the chorion facing the uterine cavity will gradually shrink. This is called the smooth chorion, surrounding the amniotic sac, fusing with it where the umbilical cord exits the placenta, forming the amnio-chorionic membrane (figure 1.5 a). The mature placenta consists of the chorionic plate on the fetal side, the basal plate on the maternal side, and between them is the intervillous space. Chorionic blood vessels from the fetal side anchor onto the maternal side through the intervillous space, where maternal blood vessels bring in nutrients and oxygenated blood to the fetus and take away waste and deoxygenated blood among other exchanges (figure 1.5 b), maternal and fetal blood never actually mix (Herrick, et al. 2022; Sapunar, et al. 2022; Douglas College, et al. 2017, Jansen, et al. 2020).

1.3.3 Trophoblast Cells, Choriocarcinoma and JEG-3 cells

As mentioned in the sections 1.3.1 and 1.3.2, trophoblast cells play a key role in placentation and even progression of pregnancy via their endocrine role of the production of hCG (Cole. 2020). They are also the main subject of our master study. As such, it is important to understand their development, differentiation, and function during placentation and pregnancy. As mentioned in the section 1.3.2, the trophoblast cells start out as the cellular layer that makes up the outer layer of the blastocyst (trophectoderm) prior to implementation in early pregnancy (figure 1.4 a) and serve as the first stage of differentiation during pregnancy (Wang, et al. 2010, Knofler, et al. 2019). The trophoblast cells in the trophectoderm consist of cytotrophoblasts (CTBs) and can be referred to as the stem cells of the placenta as they are the progenitor cells from which every other trophoblast linear differentiates (figure 1.6). Starting at the implementation of the embryo into the uterine wall, CTBs either proliferate and invade the maternal endometrium as extravillous cytotrophoblasts (EVCTs) or differentiate into villous trophoblasts (VTs) which then fuse into the syncytiotrophoblast (ST), both with their distinct roles and functions (figure 1.6) (Tarrade, et al. 2010). The EVCTs are responsible for the implementation and anchoring of the embryo into the maternal endometrium and they do so by invading and degrading the endometrium and maternal blood vessels via the excretion of proteases (Chang, et al. 2019; Salamonsen. 1999). This process leads to the remodelling of muscular and vascular tissue of the maternal uterine wall which allows for the direct exposure and bathing of the trophoblast cells in maternal blood and later the formation of the chorionic villi through which fetal blood makes exchanges with maternal blood circulating in the intervillous space (figure 1.5) (Silva, et al. 2016). As mentioned earlier, CTBs also differentiate into villous trophoblasts (VTs) which then fuse into syncytiotrophoblast (ST; Tarrade, et al. 2010). The ST plays an important role in the maintenance and progression pregnancy as mentioned in the section 1.3.1. As the EVCTs invade and prepare the uterine tissue for the formation of the placenta, CTs differentiate into VTs and fuse into ST which then expands and erodes maternal blood and surrounds lacunae filled with maternal blood. The chorionic villi develop from VCTs which invaginate and form these root-like structures within the remodeled uterine wall surrounding the pockets of maternal blood which eventually form the intervillous space (Silva, et al. 2016). As mentioned in the section 1.3.2, by week 16 of gestation, placentation is largely complete and from here on the trophoblast cells will continue to do their respective roles with the EVCTs further invading the uterine cavity and even maternal blood vessels to allow for the chorionic villi to expand and proliferate to accommodate the needs of the growing fetus (Silva, et al. 2016; Guibourdenche, et al. 2010). More details on the structure of the chorionic villi and individual trophoblastic tissues can be found in figure 1.7. One of the primary characteristics of

EVCTs is their capacity for proliferation and invasion which is crucial for the anchoring of the placenta and formation of the chorion (section 1.3.2), and interference of these processes can lead to fetal growth restriction, preeclampsia, and even miscarriage (Silva, et al. 2016). This capacity for invasion and proliferation is also a hallmark characteristic of cancer where its capacity for metastasis, among other traits, is used to differentiate a malignant tumor from a benign one (Fares, et al. 2020). Choriocarcinoma is a cancer that's derived from trophoblastic cells and appears in two forms. Non gestational choriocarcinoma is derived from germ cell tumors and is not relevant to us. Gestational choriocarcinoma can arise from normal pregnancies but most often following a miscarriage but can also arise from hydatidiform moles which are nonviable fertilized eggs that got implanted in the uterus which results in choriocarcinoma in 1 in 40 patients (Bishop, et al. 2023; Ghassemzadeh, et al. 2023). Choriocarcinoma occurs in 1 in 40,000 in north America, and as frequently as 1 in 2882 pregnancies in China with other risk factors being advanced maternal age, contraceptive use, and having a blood type A (Bishop, et al. 2023; Lurain. 2010). Gestational choriocarcinoma arises from CTBs that become malignant. These CTBs even further differentiate into VTs and ST which then mimics the development of development of a normal blastocyst as discussed above and in the section 1.3.2 (Bishop, et al. 2023).

JEG-3 cells are a choriocarcinoma derived secondary cell line that is used as an in vitro model to study the early placenta. They have strong proliferative and invasive properties like early EVCTs in the first trimester of pregnancy, so they make a good comparative model since they share those characteristics and are derived from the same tissue (Rothbauer, et al. 2017; Cai, et al. 2016).

a

b

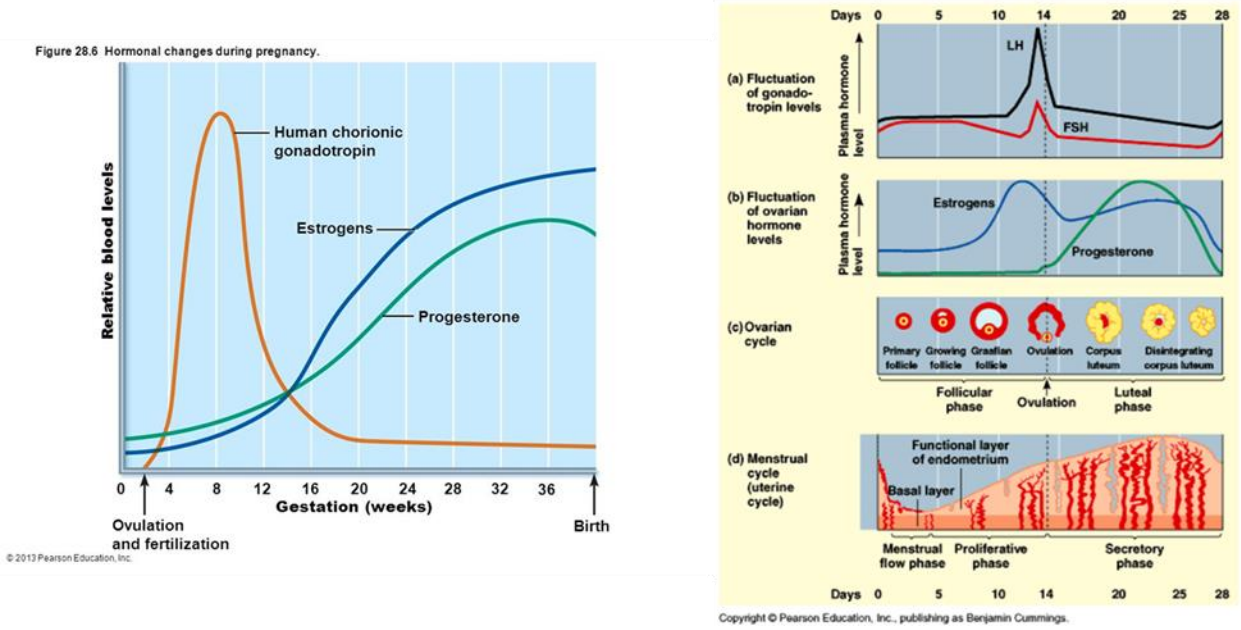

Figure 1.3: Hormonal levels following fertilization (a) and when fertilization fails to take place (b). (a) Human chorionic gonadotropin peak in the first trimester following fertilization helps maintain estrogen and progesterone levels by maintaining the corpus luteum. (b) in the absence of fertilization, progesterone and estrogen levels drop following the LH (and FSH) peaks, leading to the disintegration of the corpus luteum, drop in progesterone and estrogen levels, and the secretion of the endometrium. (Reece, et al. 2012)

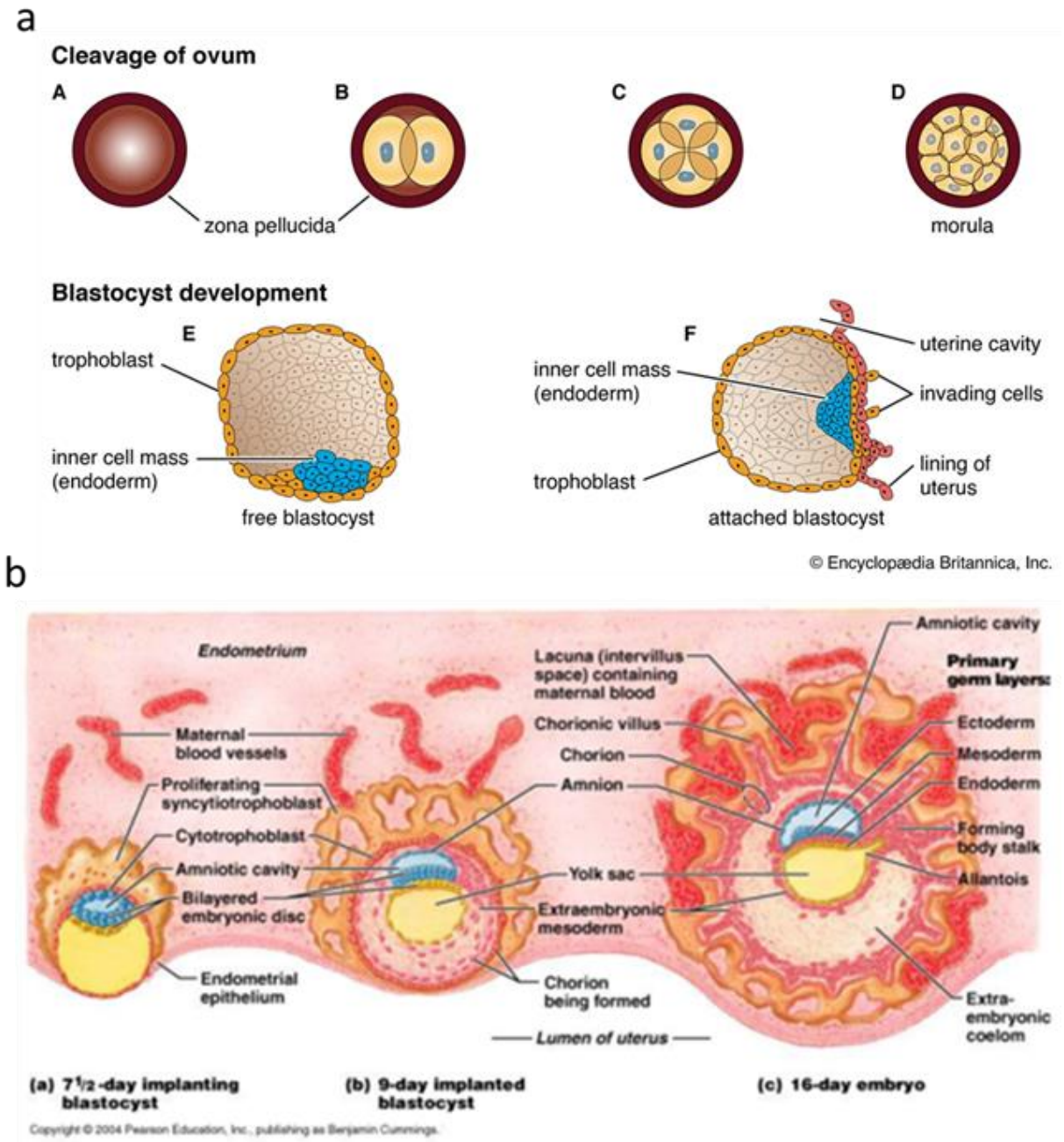

Figure 1.4: Placental formation from blastocyst to the chorionic stage. (a) following cleavage, the embryo forms the inner cell mass and trophoblast which invades the maternal endoderm. (b) the syncytial trophoblast progressively anchors itself into the endoderm, until the embryo is fully buried in the endoderm. It gradually branches out, bathing in maternal blood forming the chorionic villi which are layered by the mesoderm (pink cells) forming the chorion (Sapunar, et al. 2022; Campbell, et al 2004)

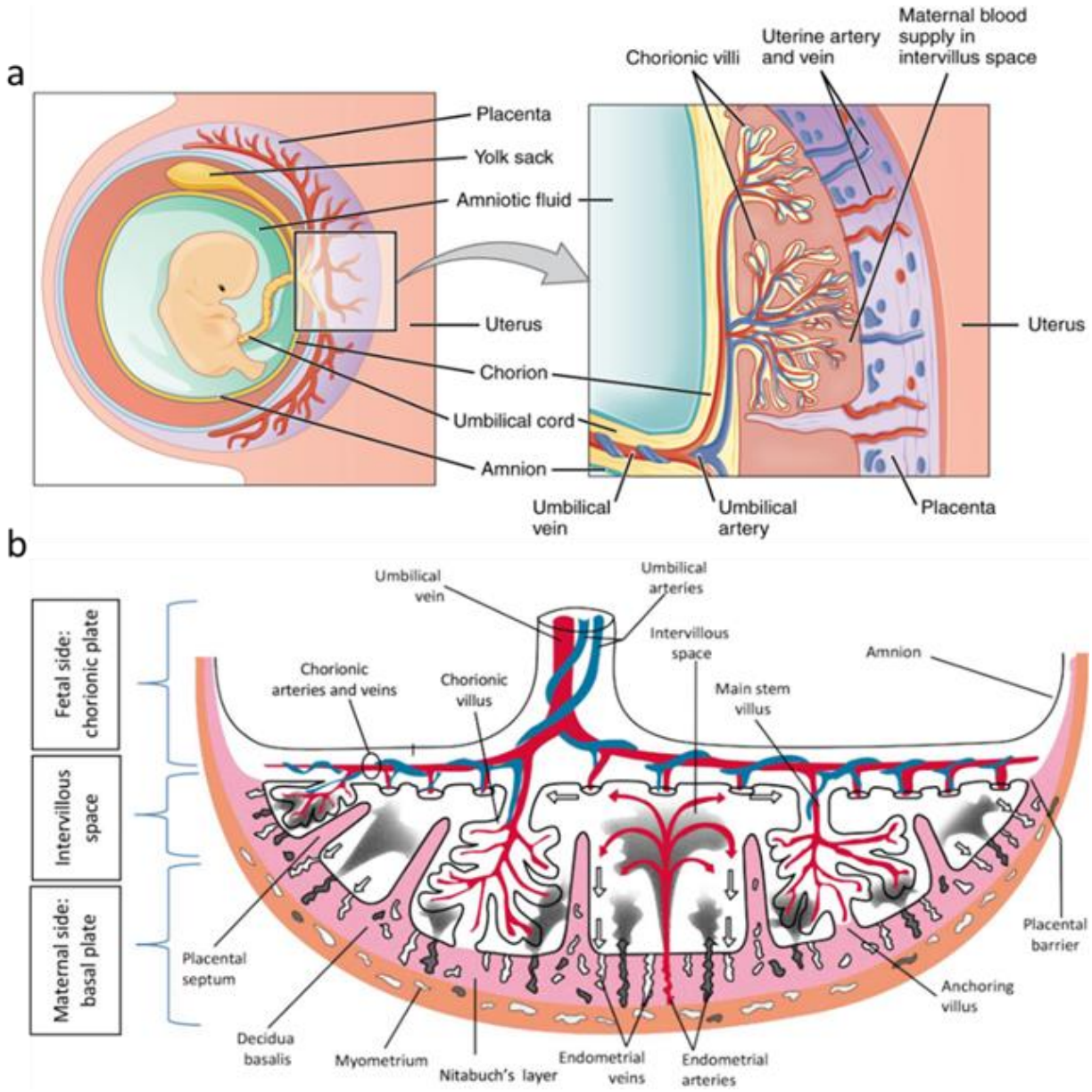

Figure 1.5: Placental and chorionic physiology post placentation. (a) smooth chorion (blue) fuses with the amniotic sac (yellow) to form the amnio-chorionic membrane. (b) material and gas exchange occur through the basal plate (endometrium) via gradients and affinity and exchanges allowing the mother to supply the offspring and remove waste without maternal and fetal blood ever mixing (Douglas College, et al. 2017, Jansen, et al. 2020)

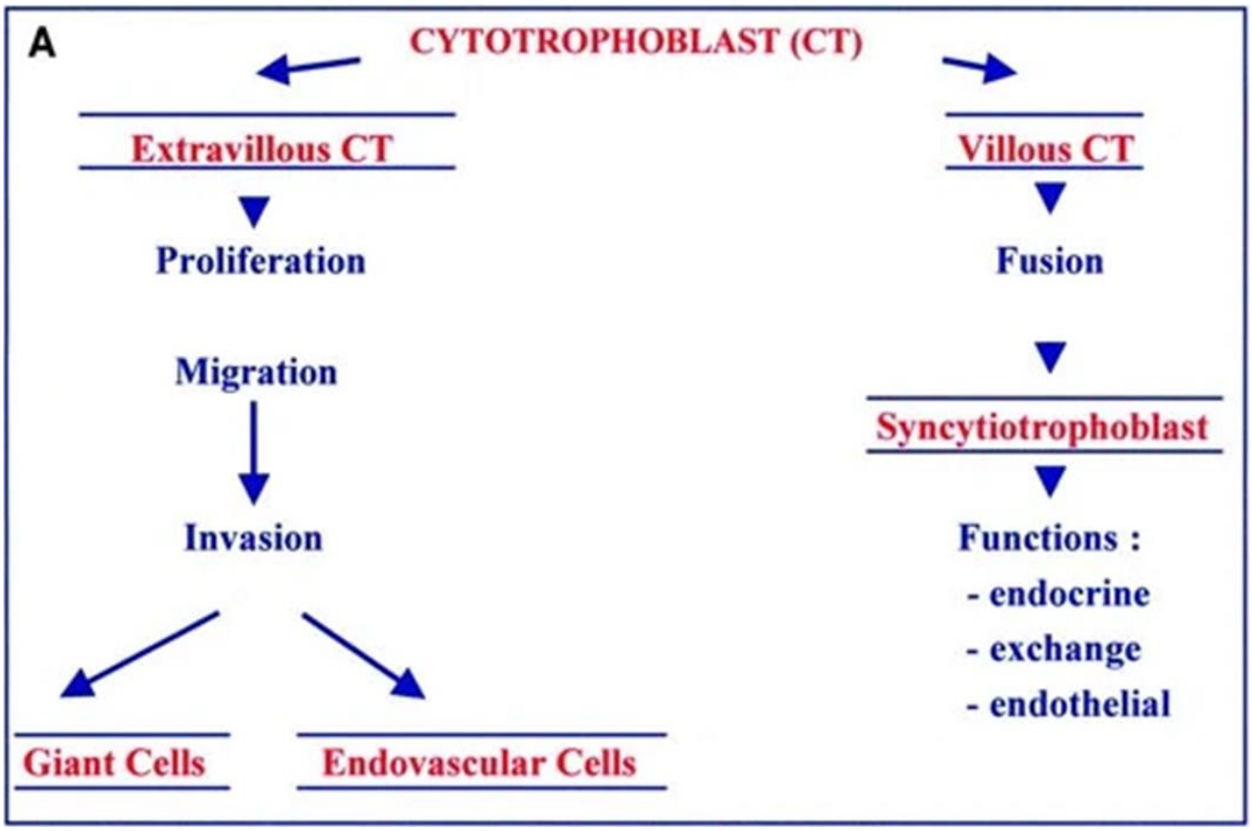

Figure 1.6: Differentiation pathway of trophoblast cells from extravillous cytotrophoblasts. (left) responsible for the invasion of the uterine wall and anchoring of the placenta, and the villous trophoblast (right) which fuse into syncytiotrophoblast and is responsible for the endocrine and exchange roles of the placenta (Tarrade, et al. 2001).

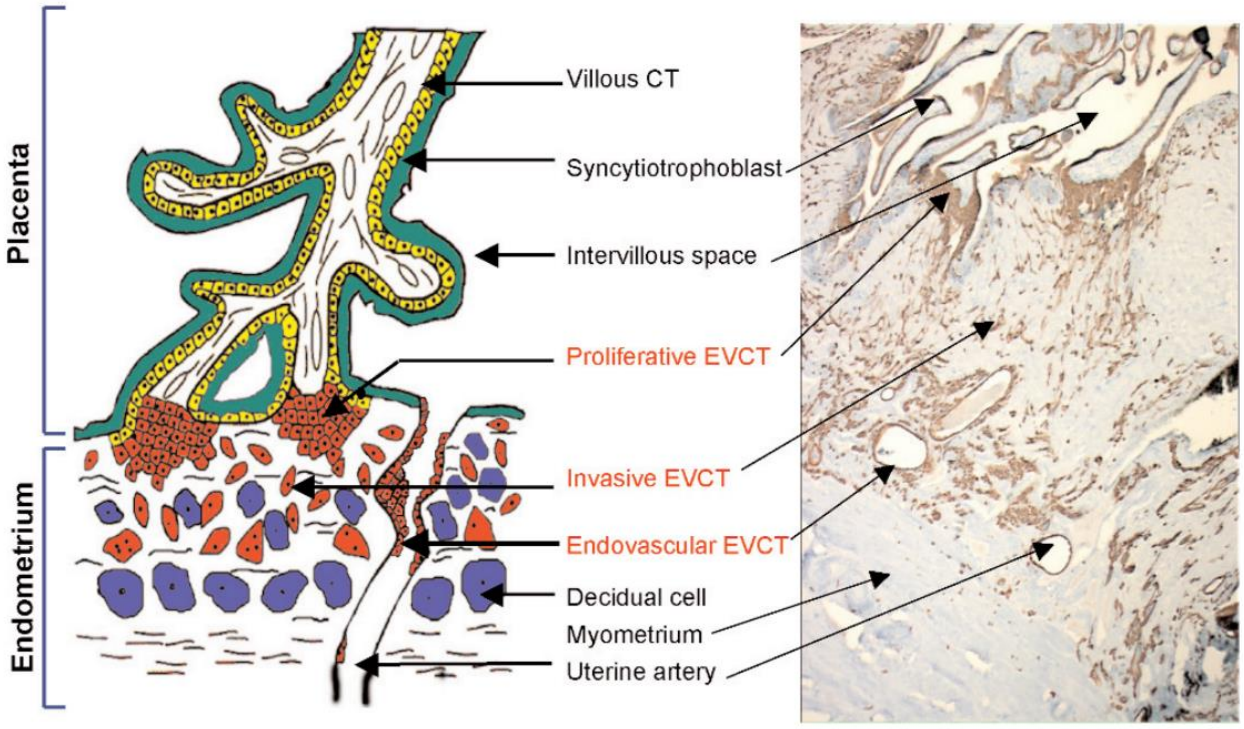

Figure 1.7: Left panel, schemes of the human chorionic villi. Right panel, immunostained section of 16-week-old human placenta following a hysterectomy diagnosed at 16 weeks of gestation. The chorionic villi are lined internally with VCTs and externally with the ST while the EVCTs continue to proliferate and invade into the maternal endometrium and even the maternal blood vessels. Material blood exchange occurs via the ST and VCTs with maternal blood occupying the intervillous space and fetal blood residing within the chorionic villi (Guibourdenche, et al. 2010)

1.3.4 The impact of endocrine disrupting chemicals on the placenta

As mentioned in the section 1.1.6, endocrine disruptors are particularly harmful during periods of high development and differentiation in an organism's life, and no period is more dynamic than embryonic and fetal development where the entire organism forms from a single cell. This fact, compounded with the placenta's important endocrine role and high sensitivity to hormonal signals, makes it a prime target of endocrine disruptors, whose impact ultimately will affect the developing offspring, leading to a wide variety of disorders that could affect the health of the offspring for the rest of its life, and even lead to a miscarriage (death of the offspring). The specific effects of EDCs on the placenta, and more specifically trophoblast cells are not fully understood, in large part due to the different types of EDCs that exist, and that even a single EDC can act on many endocrine pathways in a multitude of ways, making it difficult to get a clear picture of not only individual affected processes, but how they all interact with each other under the influence of EDCs (Gingrich, et al. 2020). Some of the more well-known EDCs in relation to placental development

and function are heavy metals such as cadmium (Cd), and lead (Pb) (Fraser, et al. 2014); plasticizers such as phthalates and its variants; xenoestrogens such as BPA, as well as UV filters such as Parabens (Yan, et al. 2023). The risk posed by these types of endocrine disruptors does not only come from their biochemical and endocrine impact on the organism, but also from the fact that they are practically ubiquitous in our modern environment, and everyone is exposed to them in some way, directly or indirectly (Yan, et al. 2023).

For example, Cd is a common component of cigarettes so exposure to cigarettes both via smoking and second-hand smoke during pregnancy can affect placental function and by consequence the fetus. It was shown that Cd can lead to lower placental weight and efficiency, which can lead to fetal growth restriction and alteration in the central nervous system (Punshon, et al. 2019). Additionally, Cd can induce inflammatory cytokine production in mouse placenta and human trophoblast cell models (Hu, et al. 2018).

Pb, if exposed to, poses a high risk to pregnancy and the developing fetus. Pb readily crosses the placental-fetal barrier and can upregulate apoptosis in the fetus as well as interfere with endoplasmic reticulum and mitochondrial function (Yan, et al. 2023; Goyer. 1990). Fortunately, exposure to Pb has been minimized in recent years after several public health scandals related to it in the past such as exposure to Pb paint, and the presence of Pb in gasoline. Unfortunately, even minimal low-level exposure can induce changes to the placenta. Indeed, a previous study showed that even low increase in Pb in maternal blood can lead to a decrease in Ca^{2+} transport in the ST which is very important for fetal survival and placental development (Lafond, et al. 2004).

Plasticizers such as phthalates and BPA, are commonly found in plastics (as their name suggests) and are. known xenoestrogens. Phthalates and BPA have both shown to inhibit placental trophoblast development, in particular, trophoblast invasive capacity and syncytialization, which interferes with proper placentation in general, disrupting the vascularization as well as leading to growth restriction. These two EDCs also cross the placental-fetal barrier, accumulating in the fetus (Yan, et al. 2023).

The Parabens, which act as UV light filters as well as preservatives in cosmetics, pharmaceuticals, and even food item, are shown to be able to cross the feto-placental barrier leading to their accumulation the fetus. They also showed to induce apoptosis in HTR8/SVneo cells, a trophoblast cell line, and reduce their proliferation (Yan, et al. 2023; Andersen, et al. 2021; Kolatorova, et al. 2017) which disrupts proper placentation which can lead to a wide variety of developmental abnormalities and even miscarriage. Another class of UV filters are metallic

nanoparticles, a class of molecules with a strong potential to act as endocrine disruptors which are found ubiquitously in modern human environment but are very poorly understood.

1.3.5 The effect of nanoparticles on the placenta

As mentioned previously (section 1.1.6), nanoparticles are an increasing public safety concern due to the rapid increase of their usage in everyday life and various industries. In the context of reproduction, and more specifically during pregnancy, nanoparticles have a strong potential to cross the maternal-fetal barrier due to their small size (Teng, et al 2021). It was demonstrated that nanoparticles as big as 500 nm in diameter can cross the placental barrier, and exposure to nanoparticles of 20 nm (200 ug/ml) and 40 nm (500 ug/ml) can induce apoptosis in trophoblast cells in mice (Huang, et al. 2015). In the context of TiO_2, ZnO, and CeO_2, several studies done on pregnant mice and rats models showed that they have a direct impact on the fetal development as well as the mother (Hong, et al. 2017; Hong, et al. 2014; Chen, et al 2022). In a mouse model TiO_2 administered orally from days 0 to 17 of gestation showed an increase in titanium content and a reduction in zinc and calcium in the maternal serum and, the placenta, as well as, reduced fetal weight and live fetuses. The same study also demonstrated a significant effect on fetal skeletal development, indicating that TiO_2 can cross the fetal barrier (Hong, et al. 2017). Specifically, nanoparticles of small diameter such 35-nm titanium dioxide nanoparticles can cross the placental barrier, enter placental cells, and induce apoptosis (Keelan, et al. 2011). The accumulation and permeability of the nanoparticle is further confirmed by a study on pregnant Sprague Dawley rats where inhaled TiO_2 nanoparticles during gestation resulted in TiO_2 nanoparticles distributed in the uterine horns and the placenta. Furthermore, the nanoparticles were detected within the cellular nucleus endoplasmic reticulum and vesicles (D'Errico, et al. 2022).

In the case of ZnO, a study done on rats demonstrated that exposure by gavage to 200 mg/kg/day and 400 mg/kg/day on gestational days 5-19 lead to reduced food consumption, and reduced body weight for the mothers and the fetus, and increased incidence of morphological abnormalities in fetuses when subjected to 400 mg/kg/day. (Hong, et al. 2014). Conversely, a study on HTR8/Svneo trophoblast cell line and isolated decidua endothelial cells demonstrated that ZnO supplementation can reduce the expression of certain inflammatory factors which allows it to modulate placental inflammation (Balduit, et al. 2020).

Finally, CeO_2 was shown, in pregnant mice via intravenous exposure, at 5 mg/kg/day and 7.5 mg/kg/day to lead to nanoparticle deposits within the trophoblast cells. It also upregulated autophagy and reduced migration and invasion of HTR8/SVneo trophoblast cell lines in vitro

(Chen, et al 2022). Furthermore, it was demonstrated in primary VT cells that CeO_2 nanoparticles are internalized by both VTs and STs (differentiated from the harvested VTs) where they upregulated apoptotic factors (caspase activation, LDH release) and also decreased the fusion capacity of VTs to ST which disturbed the secretion of vital pregnancy hormones like hCG, and hPL as discussed in the section 1.3.1. (Nedder, et al. 2020). Lastly, as mentioned in section 1.3.5 CeO_2 is a component of catalytic convertors and as such ubiquitous in its usage and presence in daily environment. An important study on the co-exposure of CeO_2 and car emission pollutant benzo(a)pyrene (B*a*P) demonstrated using chorionic villi explants and harvested VTs that CeO_2 modulated the effect of B*a*P with regards to genotoxicity and an antioxidant effect both individually and co-exposed indicating that the effects of CeO_2 are not straight forward (Deval, et al. 2023).

2 Hypothesis and Objectives

2.1 Problematic

The COVID-19 pandemic and its public health measure led to unprecedented disturbances to human activity and changes in human behavior in recent history. These changes range from personal habits with regards to the usage of cleaning and sanitation products, and the use of single-use plastic and personal protective equipment, to the shut down of entire sectors of society (namely commercial and entertainment). Such a radical shift in human behaviours is bound to have an effect both environmentally and on human health.

Several studies observed that a drastic increase in the use of sanitary and cleaning products single-use plastics, and personal protective equipment such as gloves and masks. This naturally led to an increased risk of exposure to harmful chemicals and even environmental contamination with a large risk posed by endocrine disrupting chemicals, especially in urban areas. On the other hand, in terms of general pollution the pandemic has had positive effects on the environment, in that reduced human activity, led to a temporary reduction in noise pollution, air pollution and even water pollution. To that end, we sought to observe how these conditions have affected the environment in Quebec, particularly in Greater Montreal, by looking at existing data on water pollution before and during the pandemic.

Our lab studies the human placenta and toxicology related to it, and as mentioned above, the pandemic led to an increased risk of exposure to endocrine disrupting chemicals, the effects of which on human health might not be readily apparent. As such, we sought to understand the effects some of these potential endocrine disruptors might have on the human placenta which could have consequences on health for generations. One such class of endocrine disruptors is metallic oxide nanoparticle, specifically, TiO_2, ZnO, and CeO_2 whose endocrine disrupting effect on placental function and development during pregnancy are poorly understood.

2.2 Hypothesis and specific objectives

Hypothesis 1: There was an increase in exposure to contaminants related to cleaning and sanitation products in the general population due to increased usage during the pandemic which would lead to an increased presence of these chemicals or their by-products in municipal wastewater.

Objective 1: to analyze municipal wastewater data before and after treatment to check the level of contamination of municipal wastewater before and during the pandemic to assess whether the pandemic had a local and/or global effect on the environment in the urban area of Montreal.

Hypothesis 2: As a result of increased exposure to endocrine disruptors found in single-use plastics and various cleaning and sanitation products there will be an increased impact on human health. Compounds such as TiO_2, ZnO, and CeO_2 found in single-use plastics and some self-care products will have a detrimental effect on placental development, function and therefore fetal health and development.

Objective 2: to test the effects of TiO_2, ZnO, and CeO_2 on placental cell model JEG-3 viability in vitro to assess whether further studies should be conducted on their effects as endocrine disrupting chemicals in the context of placental development, health, and pregnancy.

3 Materials and Methods

3.1 PortailEauQuebec and AtlasEau

We accessed wastewater data via “Portail des connaissances sur l’eau” (PCE) which is a website by the Quebec “Ministère de l'Environnement, de la Lutte contre les changements climatiques, de la Faune et des Parcs” (https://www.environnement.gouv.qc.ca/eau/portail/). The data is collected by the water treatment stations across the province, under the SOMEAU system (https://www.environnement.gouv.qc.ca/eau/eaux-usees/somaeu/fiche_no4_somaeu.pdf). This includes both affluent and effluent data, referring to water data before and after purification respectively, only the latter of which gives us unbiased data on water contamination. The stations collect data on water flow, acidity, nitrate content. In this study, our primary focus is on “MES (Matière en Suspension), DCO (Demande Chimique en Oxygène), and DBO5C (Demande biochimique en Oxygène 5 jours, Partie carbonée)”.

The “AtlasEau” (https://www.environnement.gouv.qc.ca/eau/atlas/index.htm) is an interactive map tool that is a part of the portal and visualizes individual water treatment stations in Quebec, allowing to individually pick and look at the data of each station in the province. Using that map and the suggestions of ministry worker Simon Pineault, we included in our study 5 stations of interest in the province that correspond to the greater Montreal area. The 5 stations are as follows: Station d’épuration: Montréal (Jean-R.-Marcotte) - (00065-1) #652; Station d'épuration : Montréal (Île Notre-Dame) - (00065-2)] #80; station d'épuration : Laval (Fabreville) - (64500-1) #646; Station d'épuration : Laval (Ste-Rose-Auteuil) - (64500-2) #215; Station d'épuration : Laval (Lapinière) - (64500-3) #651 (figure 3.1). Station Jean Marcotte is the principal station for Montreal serving over two million people. Notre Dame Island is unique in that it exclusively serves as a commercial and touristic area (which contains the La Ronde amusement park). Fabreville is also interesting in that it receives water from the north of Quebec. The other two stations just serve their local areas.

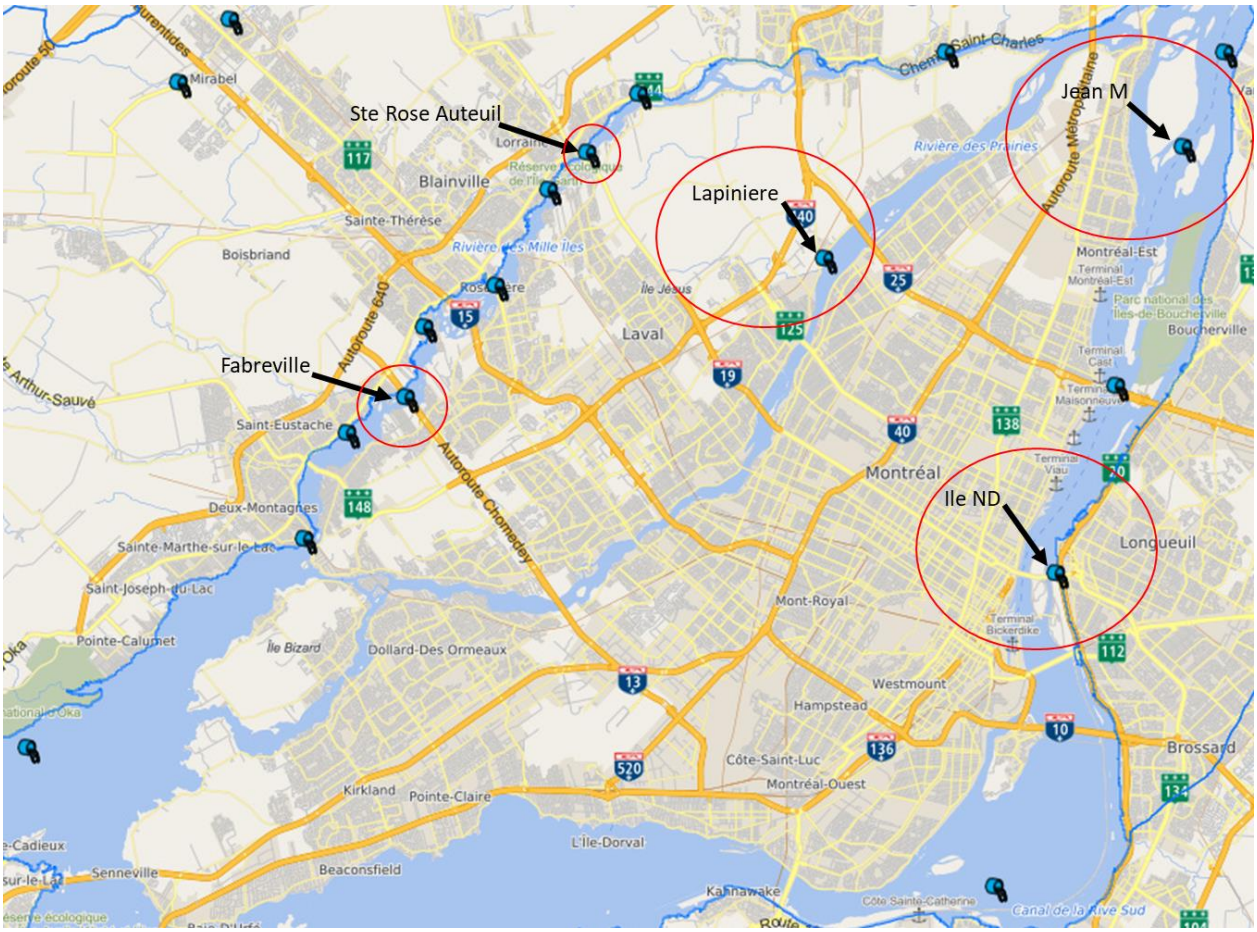

Figure 3.1: AtlasEau map zoomed in to Greater Montreal. Highlighted are the 5 stations of interest in this study: Fabreville, Sainte Rose Auteuil, Lapinière, Jean R Marcotte (Jean M), and Ile Notre Dame (Ile ND) (https://www.environnement.gouv.qc.ca/eau/atlas/index.htm).

3.1.1. Portal Data analysis

Raw data was compiled in excel spreadsheets by purification station. We used R software to extract the relevant data for the 5 stations of interest and then organized the data for each station chronologically from 2017 to 2021. We then used GraphPad Prism v9.4.0 (GraphPad Software, San Diego, CA). to process the data year by year to check for any variation in how water contamination changed during COVID, and its lockdowns compared to the pre-COVID years. analyzed the data with respect to the officially declared “waves” of the pandemic as described by the Institut national de la santé publique du Québec (INSPQ) (figure 3.2). Additionally, we looked for periods of environmental disturbances related to water that might affect municipal wastewater.

To that end, we used two periods of flooding that took place in Quebec in between April and May of 2017 and 2019 (Lau. 2017; Saint-Arnaud. 2019). The data analyzed pertains to three categories of contamination and since the data was collected by the government of Quebec it is abbreviated in French as follows: DCO – demande chimique en oxygène (Chemical oxygen demand; COD)), DBO5C - Demande biochimique en oxygène à 5 jours partie carbonée (biochemical oxygen demand over 5 days carbonaceous demand; DOD5C), and MES - Matière en suspension (total suspended solids ; TSS).

Vague 1	Vague 2	Vague 3	Vague 4	Vague 5
25 février 2020 au 11 juillet 2020	23 août 2020 au 20 mars 2021	21 mars 2021 – 17 juillet 2021	18 juillet 2021 – 4 décembre 2021	5 décembre 2021 – 12 mars 2022

Figure 3.2: COVID-19 pandemic waves according to the INSPQ. Each wave corresponds to bouts of increased and then decreased infection cases (INSPQ, 2022).

3.2 Effect of nanoparticles on human trophoblast cell

3.2.1 Materials

Nanoparticles titanium dioxide (109TM), zinc oxide (111ZO), and cerium dioxide (101CO) were purchased from Scieventions Inc. (Toronto, Canada) All well plates, and T-75 flask were purchased from Corning Inc. (Corning, NY). EMEM 1X media, containing Earle's salts and L-glutamine (320-005 CL) was purchased from Wisenbiproducts (QC, Canada). The 10% fetal bovine serum (FBS) was purchased from Multicell (Woonsocket, RI). 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was purchased from Sigma (St. Louis, MO) and 100 mM sodium pyruvate was purchased from ThermoFisher (Waltham, MA). 0.4% Trypan blue was obtained from Sigma (St. Louis, MO), and TC10 cell counter and the dual-chamber cell counting slides purchased from BioRad (Hercules, CA). The incubator used throughout the entire experiment was purchased from ThermoFisher (Waltham, MA). The Zetasizer Nano ZS (model ZEN3600) was purchased from Malvern Pananalytical (Westborough, MA) Phosphate Buffered Saline (PBS) and Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma. TryLE^{tm} was purchased from ThermoFisher. SpectraMax M5 spectrophotometer was purchased from Molecular Devices (San Jose, CA). The eBioscience Annexin-V apoptosis detection kit was purchased from Invitrogen (Walthham, MA) and included annexin V FITC, propidium iodide (PI) and a binding buffer. 1 µM staurosporine was purcahsed from Cell Signalling Technologies (Danvers, MA). CellQuest Pro software, Becton Dickinson FACSCalibur flow cytometer, and the

FlowJo v.10 software were all purchased from BD Biosciences (Franklin Lakes, NJ). Circular cell adherent coverslips (12-545-102) and microscope slides were obtained from Fisher Scientific (Waltham, MA). Calcium chloride (10043-52-4; $CaCl_2$), and ammonium chloride (12125002-9; NH4CL) were purchased from Sigma, and magnesium chloride (7786-30-3; $MgCl_2$ and 4% PFA was purchased from Acros Organics (Waltham, MA), Triton X-100 from Sigma, Hoechst dye and the ProLong Gold Antifade Mountant were purchased from ThermoFisher The microscope used is the Ecplise E800 from Nikon (Melville, New York).

3.2.2 JEG-3 cell culture

JEG-3 cell line was purchased from American Type Culture Collection (ATCC, 2023). The cells were incubated in EMEM 1X media, with Earle's salts, and with L-glutamine. Media was supplemented with 10% FBS, 1% 100 mM sodium pyruvate solution for a final concentration of 1 mM and 1% 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer for a final concentration of 10 mM. Cells were incubated at 37ºC and 5% CO_2 in T75 flasks. The cells were split in a 1:4 ratio once they reached 80% confluency, no higher than passage 30.

Cells were counted using 0.4% trypan blue in a 1:1 ratio using the TC10 automated counter. All cells were given a 24h recovery period prior to any treatment of manipulation to allow cells to adhere to their container and revitalize following trypsin treatment.

3.2.3 Nanoparticles characterization

The nanoparticles TiO_2, ZnO, and CeO_2 were characterized in terms of size and charge. The size distribution, surface charge (zeta potential) and the polydispersion index (PDI) of the nanoparticles were determined by the dynamic light scattering using a Malvern Zetasizer Nano-ZS. They were measured between 1nm to 1000 nm. Two controls were set. Nanoplastics which were used as a control for nanoparticles since nanoplastics are neutral and have a low tendency to form large aggregates. To control for the effects of the cellular media, the nanoparticles were also measured in MiliQ water. The nanoplastics and the nanoparticles were also tested in the cell media discussed above.

3.2.4 MTT viability assay

The assay was carried in clear, flat-bottom 96-well plates. Cells were seeded at a concentration of 5.0×10^3 cells per well, 100 µl of culture per well (5.0×10^4 cells/ml). Following a 24h incubation period, the cells were treated with increasing concentrations of TiO_2, ZnO, and CeO_2 from 0 mg/ml to 1000 mg/ml in intervals of 100 mg/ml for a period of 24h and 48h and incubated as usual. Following treatment period, an MTT solution in PBS was added and the cells were incubated for

4h in the same conditions as mentioned in the section 3.2.2. The formed formazan crystals were dissolved by adding 150 µl of DMSO solution with 2% glycine at pH 11. Absorption for each well was read at λ = 570 nm using the SpectraMax M5 spectrophotometer to derive the viability curves for each nanoparticle treatment by normalizing readings to 100% to untreated cells (well with cells containing only media) and controlling for the phenol in media (wells without cells and containing only media). The curves were used to determine the IC_{50} value for each nanoparticle. The assay was carried with 3 biological replicates each with 3 technical replicates.

3.2.5 Flow Cytometry

The cells were seeded in 6-well plates at a concentration of 1.0 x 10^5 cells per well, 1 ml culture per well. Cells were treated for 48hr with each nanoparticle in 3 biological replicates at the IC_{50} concentration as determined in the MTT viability assay. The flow cytometry assay and analysis were done as per the manufacture protocol of Invitrogen eBioscience Annexin V apoptosis detection kit. Following treatment, the entire culture from each well was collected including the supernatant and washing buffers such as PBS. The cells were centrifuged for 5 minutes at 10,000 rpm and resuspended in binding buffer that is part of the kit. Then the cells were stained with Annexin V diluted 1:40 (part of the Invitrogen apoptosis detection kit), and then with 0.95 µg/mL propidium iodide (PI) (part of the Invitrogen apoptosis detection kit). Positive control consisted of cells incubated with 1 µM staurosporine for 24h, and no treatment controls were used as negative controls. Negative controls were used to determine quadrant boundaries using CellQuest Pro software for the Becton Dickinson FACSCalibur flow cytometer which was then analyzed using FlowJo v.10 software.

3.2.6 Immunocytochemistry (ICC) and imaging.

Coverslips mentioned in section 3.2.1 were washed with 0.1 M HCl and 70% EtOH and then placed in 24-well plates. Cells were seeded at 5.0 x 10^4 cells per well, at 0.5 ml of culture per well (1.0 x 10^5 cell/ml). Cells were treated similarly with IC_{50} concentrations of TiO_2, ZnO, CeO_2, and 1 µM staurosporine for positive control, and untreated cells served as a negative control. The cells were assayed with 3 biological replicates.

The cells were then washed with PBS Ca^{2+}/Mg^{2+} and fixed with 4% PFA for 20 minutes at RT. The cells were incubated with 50 mM NH_4CL solution at RT for 10 minutes to neutralize PFA autofluorescence. Cell membrane permeabilization was done with 0.3% Triton X-100 for 10 minutes at RT. Cellular nuclei were stained using 1 ug/mL Hoescht dye. The coverslips then were mounted on slides using ProLong Gold Antifade Mountant.

The images were obtained with a Nikon eclipse E800 microscope under 20X magnification for a total magnification of 200X, with fluorescence setting of λ_{ex} = 330nm – 380nm.

3.3 Statistical analysis

3.3.1 PortailEauQuebec Data

The data was processed to yearly data of individual stations and separate contamination sources using R software v4.1.0 and v4.2.1 to compile it by data from 2017 to 2021 for each individual stations. It was then exported to GraphPad Prism v9.4.0 (GraphPad Software, San Diego, CA) where it was further processed into a presentable format. The data for Fabreville station and Jean-Marcotte station were both averaged on a biweekly basis, whereas the data for Ile Notre-Dame was shown as is. Figures 4.2 and 4.5 show a yearly average.

3.3.2 Nanoparticle study data

The MTT viability assay was done with three biological replicates each having three technical replicates. All data was analyzed using GraphPad Prism v9.4.0. The data was normalized with no treatment treated as 100% viability. Outliers were identified for each set of technical replicates using a Grubbs' test with an alpha value of 0.2. the technical replicates were then averaged for each biological replicate and the same processes was repeated.

Flow cytometry, the viability data was averaged between 3 biological replicates to represent a means viability value using the same Grubbs' test with alpha value of 0.2.

4 Results

4.1 Analysis of the municipal water contamination data

To determine the effect the COVID-19 pandemic had on water contamination due to public health measures (lockdowns) we analysed water contamination data prior to treatment to determine levels of contamination before, during, and after the lockdowns during the COVID-19 pandemic.

4.1.1 Station Jean Marcotte as the reference point

As mentioned in the section 3.1 we analyzed the data of Station Jean R Marcotte since it is the principal station for Montreal serving over 2 million people and its data is the most comprehensive. Thus, we used it as our principal reference point. We put a specific a specific emphasis on looking for changes in contamination as they relate to the COVID-19 infection waves as shown in figure 3.2. As shown in figure 4.1, we can see a sharp drop in water contamination of DCO, MES, and DBO5C in 2017 and 2019 between March and May of these years (Figure 4.1 a, c, f, h, m) which corresponds to the time of the flooding between late February and early April of those years which is mentioned in the section 3.1.1. The one outlier is contamination related to MES in 2017 where the contamination didn't experience such a significant drop (figures 4.1 k). As expected, in 2018 which is pre-pandemic year without flooding, the contamination levels remain relatively uniform (figure 4.1 b,g,l). Then in 2020 when it can be observed another sharp decline of contamination of DCO, MES, and DBO5C around the time of the initial complete lockdown which took place on March 13th followed by a resurgence of contamination levels in the middle of April (Figure 4.1 d,i,n). Interestingly, we do not observe a similar drop in contamination in October when the second complete lockdown was initiated, from then, the contamination level remain relatively stable throughout all of 2021 (figure 4.1, d,e,i,j,n,o). In addition, we compared the wastewater contamination yearly averages at the station Jean R Marcotte. DCO levels remained stable throughout 2017 until 2019 but started to gradually increase in 2020, with an even bigger increase in 2021 (figure 4.2 a). On the other hand, DBO5C shows an incremental increase every year from 2017 to 2021 (figure 4.2, b). Lastly, MES maintained roughly the same levels over the 5-year period (figure 4.2, c). Overall, the changes in contamination are not statistically significant during the period studied, but the level of contamination for DCO and DBO5C are worth studying for the following years to see if the same trend continues which might be indicative of a yet unknown phenomenon.

Stations Jean R Marcotte, Lapinière, and Ste-Rose-Auteuil showed a similar pattern of contamination with a drastic drop in contamination for all three categories from the beginning of the lockdown in March 2020.

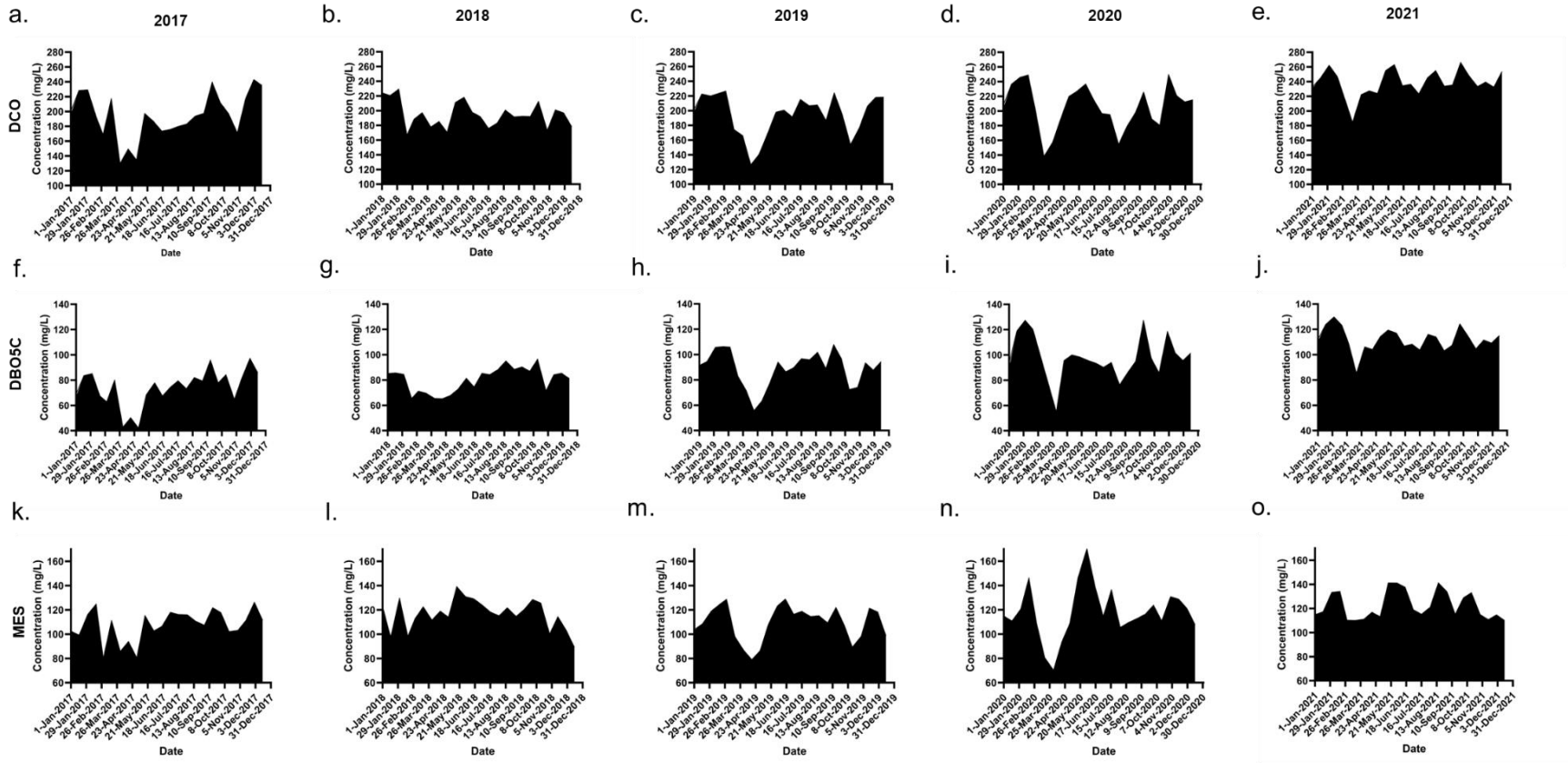

Figure 4.1: Yearly biweekly average of water contamination data from 2017 to 20121 for station Jean R Marcotte.

Data is shown for Chemical oxygen demand (DCO; first raw), biochemical oxygen demand over 5 days carbonaceous demand (DB05C, second raw), Suspended Materials (MES; third raw) from 2017 to 2021 (left to right). The data shows that contamination levels are seemingly affected by disruptions of human activities and water circulation such as the floodings that occurred in 2017 and 2019 as discussed in section 3.1 (a,c,f,h,k,m). Furthermore, the initial lockdown of the pandemic appears to have a similar effect of reduced contamination which then does not reoccur in following lockdowns especially not to the same intensity.

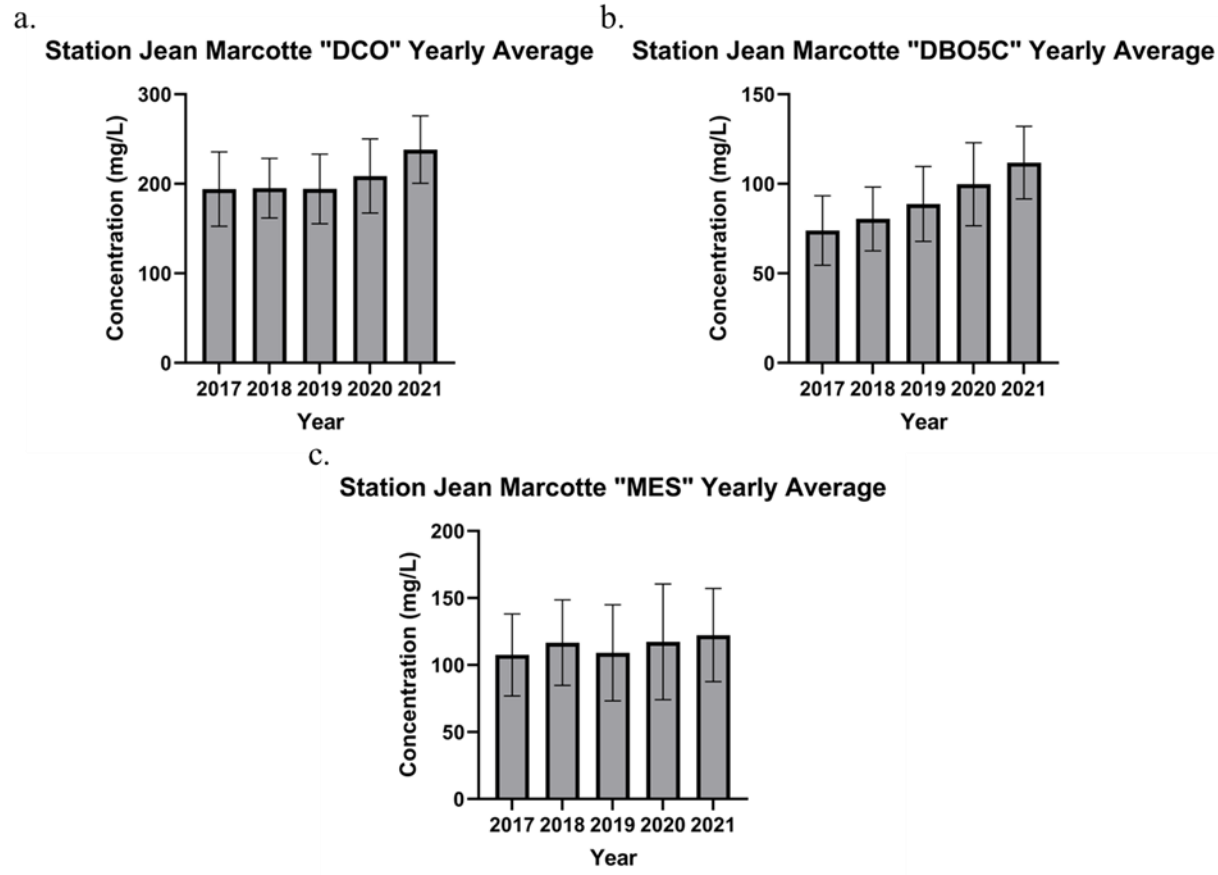

Figure 4.2: Yearly averages of water contamination data for station Jean R Marcotte from 2017 to 2021.

Data shows the averages for (a) Chemical oxygen demand (DCO), (b) biochemical oxygen demand over 5 days carbonaceous demand (DBO5C), (c) Suspended Materials (MES) contamination. Despite the increase in contamination observed for DCO and DBO5C those changed are not significant.

On the other hand, Station Île Notre-Dame showed a very different pattern. Unlike Station Jean Marcotte, Lapinière, and Ste-Rose-Auteuil, there was a drastic drop in contamination for all three categories from the beginning of the lockdown, and that contamination has never shown any recovery anywhere near to pre-lockdown levels (figure 4.3). This was not observed in any of the other four stations and likely has to do with the fact that the station primarily serves the island after which it is named, which is mainly an area of commerce and tourism, it hosts the La Ronde amusement park, activities which as of the end of 2021 were not fully re-established. As a result, there never was an opportunity for pollution and contamination levels to recover since humanity activity in the region remained low as of the end of 2021 which is reflected in the low contamination levels observed in figure 4.3.

Lastly, station Fabreville showed a relative uniform level of contamination with regular peaks and valleys of contamination between 2017 and 2020 whereas in 2021 it showed a stark increase towards the beginning of the summer of 2021 as seen in figure the 4.4. when we looked at the yearly average contamination for all three categories we saw as much as a doubling of total average value in 2021 compared to previous years (figure 4.5), which further confirms our initial observation looking at the data in the figure 4.4. As discussed in section 3.1, the station Fabreville receives water from the north of Quebec and late spring of 2021 was when the province started to fully lift COVID-19 restrictions in conjunction with the progression of the vaccination campaign (Table 1).

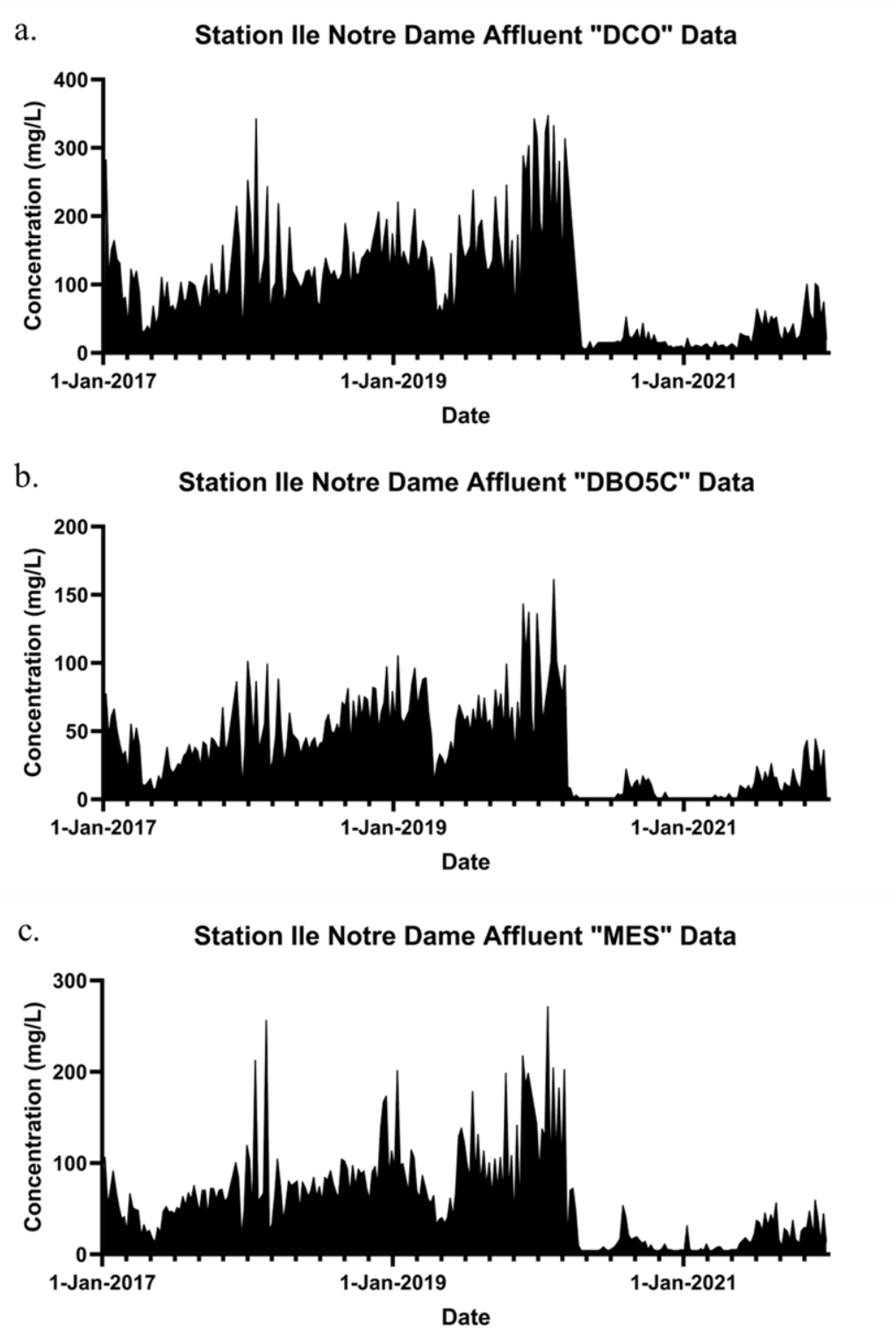

Figure 4.3: Station Île Notre-Dame water contamination data from 2017 to 2021

Data is shown for (a) Chemical oxygen demand (DCO), (b) biochemical oxygen demand over 5 days carbonaceous demand (DBO5C), (c) Suspended Materials MES showing a virtual collapse of water contamination that never recovered to pre-pandemic levels as far as the end of 2021. Data is subdivided into 2-month intervals.

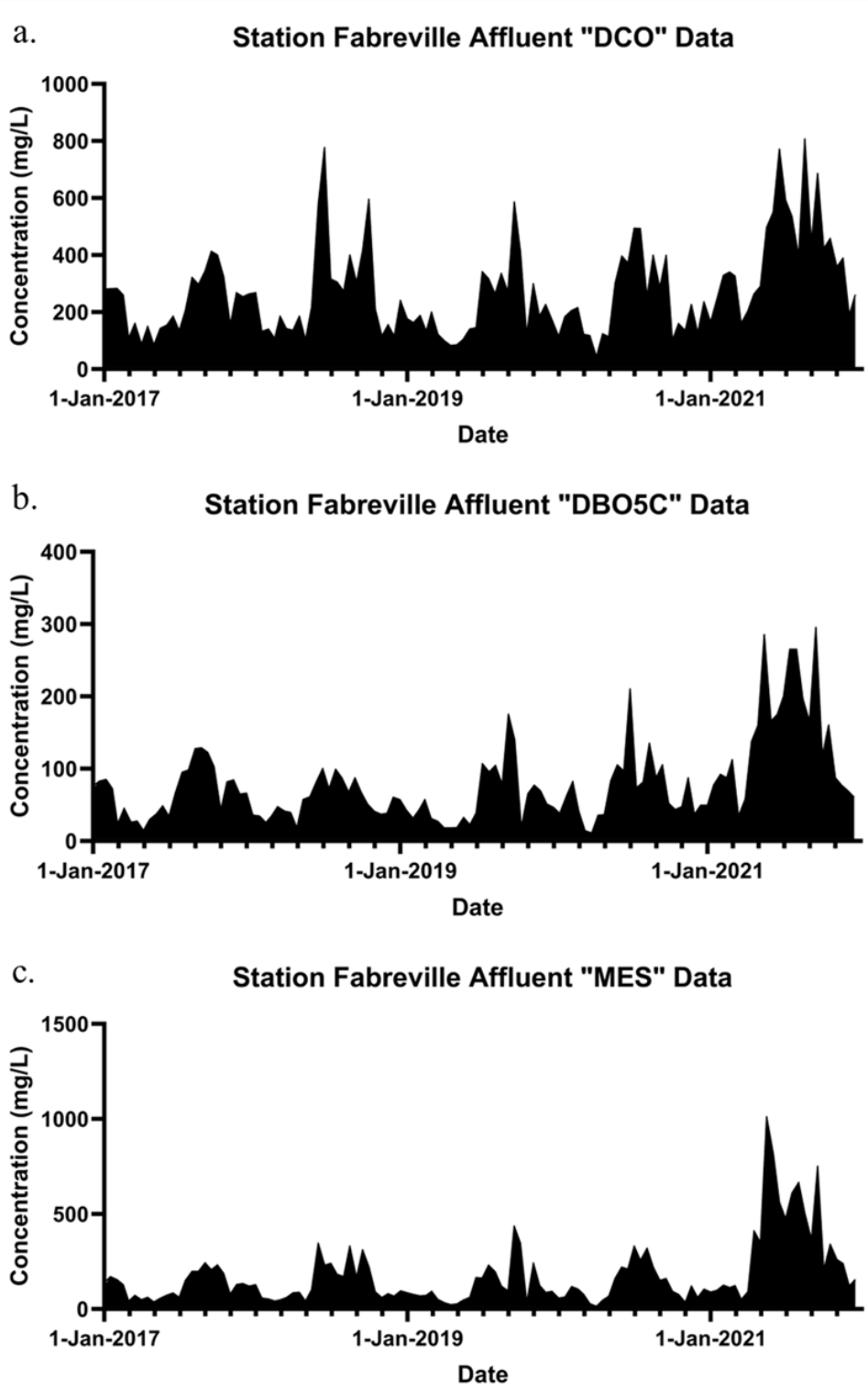

Figure 4.4: Bi-weekly averages of water contamination between 2017 to 2021 for station Fabreville.

Data is shown for (a) Chemical oxygen demand (DCO), (b) biochemical oxygen demand over 5 days carbonaceous demand (DBO5C), (c) Suspended Materials (MES) and indicates a large increase in water contamination levels that surpasses pre-pandemic levels starting in the second quarter of 2021. Data is presented in 2-month intervals.

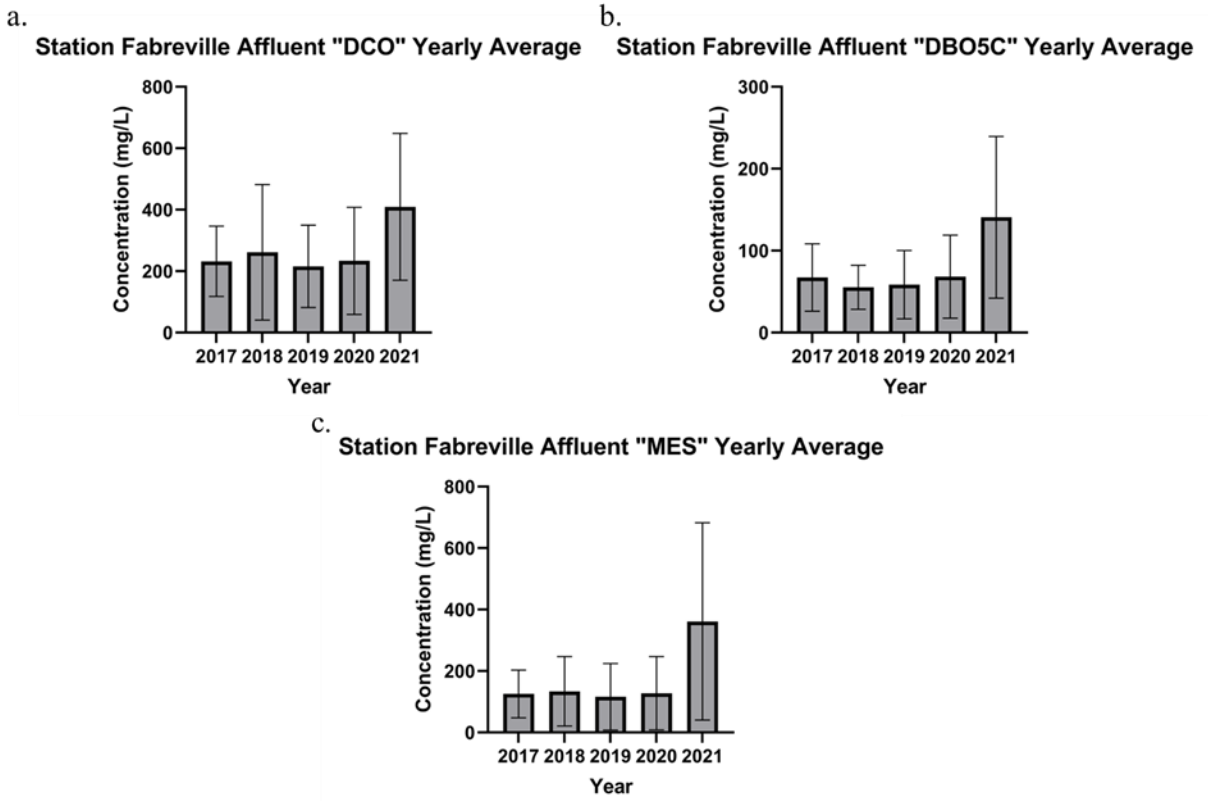

Figure 4.5: Yearly average water contamination for station Fabreville between 2017 to 2021.

Data is shown for (a) Chemical oxygen demand (DCO), (b) biochemical oxygen demand over 5 days carbonaceous demand (DBO5C), and (c) Suspended Materials (MES) a large increase in water contamination in 2021 compared to previous years.

4.2 Effects of the TiO_2, ZnO, and CeO_2 metallic nanoparticles on placental cell model JEG-3 in vitro.

4.2.1 Nanoparticle Characterization

All three nanoparticles had their charges fully neutralized in media as determined by the zeta potential test discussed in section 3.2.3, which led to some reduction in aggregation stability (more variation in aggregation sizes; figure 8.3) but the aggregate sizes were still within workable parameters (between 1 nm to 1000 nm in size), with TiO_2 having an average size of 422.7 nm, 32.84nm, and 9.642; ZnO 12.93 nm and 61.92 nm; and CeO_2 13.66 nm and 89.05 nm as per our measurements using dynamic light scattering as discussed in the section 3.2.3 (figure 8.2).

4.2.2 MTT Viability assay

JEG-3 cell viability was determined by MTT that measures mitochondrial dehydrogenase activity as it converts MTT to formazan (Ghasemi, et al. 2021). ZnO showed a very steep decline in

viability, starting at 100 µg/ml and reaching zero viability at 300 µg/ml/ml (Figure 4.6 b,e) while TiO_2 and CeO_2 showed a gradual decline in viability for both 24hrs and 48hrs exposure periods (Figures 4.6 a,c,d,f,). As such, ZnO showed a much lower IC_{50} value of 153.2 µg/ml and 146.5 µg/ml for 24hrs and 48hrs treatment respectively compared to TiO_2 which showed an IC_{50} of 963.4 µg/ml and 836.3 µg/ml and CeO_2 showing IC_{50} 903.2 µg/ml and 772.2 µg/ml for 24hr treatment and 48 treatments. respectively. Based on these values we chose the following concentrations for further analysis: IC50: TiO_2: 835 µg/ml, ZnO: 150 µg/ml, CeO_2: 775 µg/ml at 48hrs of treatment only.

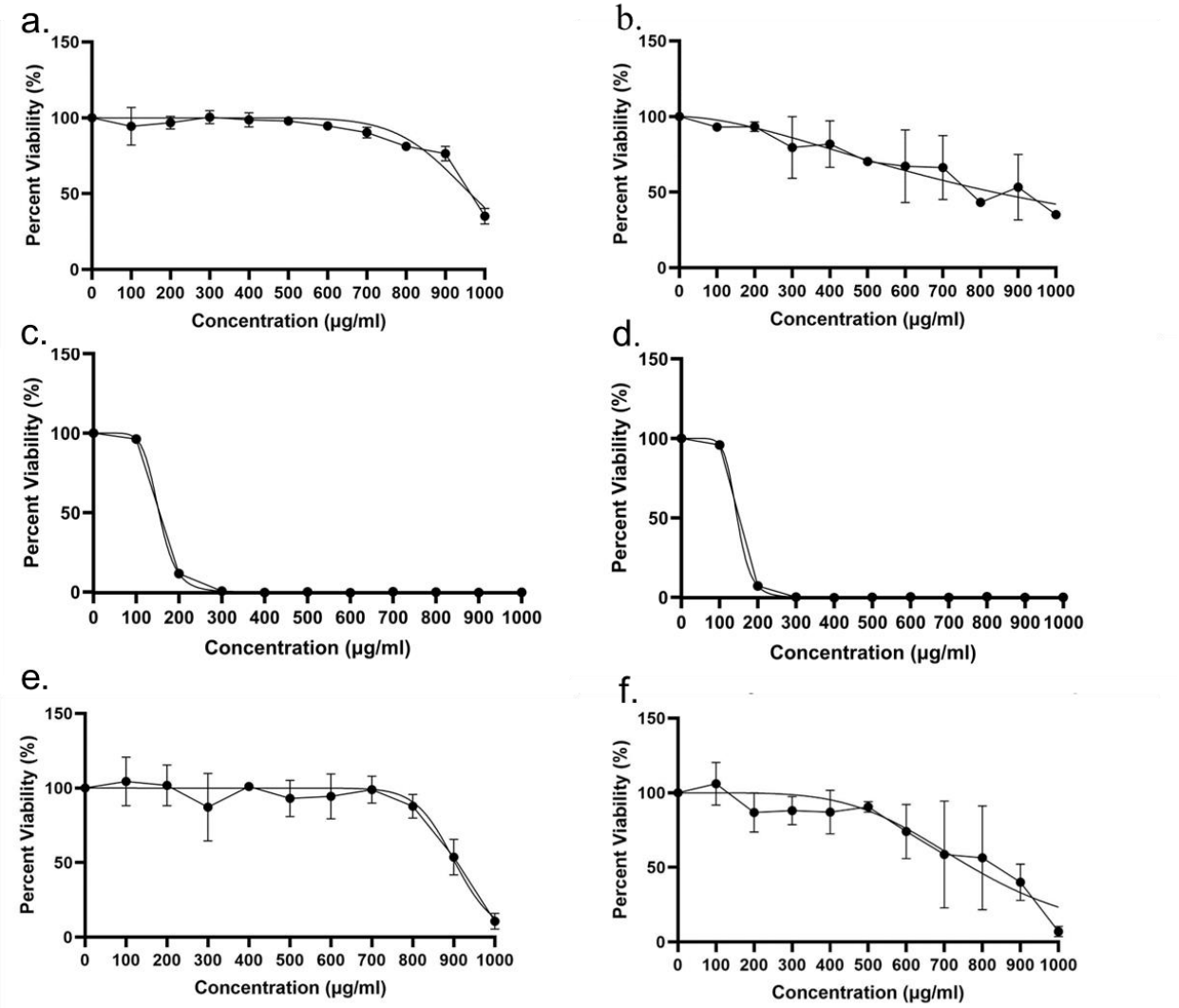

Figure 4.6: MTT assay viability curves of JEG-3 cells in response to nanoparticle exposure.

Graphs showing viability of JEG-3 cells under nanoparticle exposure to TiO_2 (top row), ZnO (middle row), CeO_2 (bottom row) at 24 hours of exposure (left column) and 48 hours of exposure (right column). Viability was normalized to the untreated cells (0 µg/ml) which act as a negative control. IC_{50} = (a) 963.4, (b) 836.3, (c) 153.2, (d) 146.5, (e) 903.2, (f) 772.2. (N = 3)

4.2.3 Effect of TiO_2, ZnO and CeO_2 on JEG-3 cell viability determined by flow Cytometry.

Flow cytometry results are expressed by individual readings of cells that are marked by fluorescent dyes that act as markers for cell death and apoptosis as described in the section 3.2.5. The flow cytometry analysis shows mixed results. We can observe a shift towards the third and second quadrant most pronounced in the JEG-3 exposed to ZnO relative to the negative control which indicates a transition into early and later apoptosis (figure 4.7). The negative control is JEG-3 cells without any treatment and the expected viability following exposure to the nanoparticles is half of the viability of the negative control. So, it acts both as a control for the lack of treatment, and the standard under which we normalize our results. However, the viability of the cells has not shifted significantly compared to the negative control when exposed to TiO_2 and CeO_2, and in the case of CeO2 it is even higher than the negative control (figure 4.8). ZnO is the only nanoparticle that leads to a significant reduction in viability, although it is not near the expected value (which would be around 45% viability compared to the 89.5% viability of the positive control; Figure 4.8). Going back to figure 4.7, despite the results shown in figure 4.8, we see a shift toward apoptosis and cellular death when exposed to each nanoparticle to varying degrees compared to the negative control, indicating that the nanoparticle have a detrimental effect on the cells, and that there might be additional factors at play that create a discrepancy between the results obtained here and what is observed with the MTT assay in the section 4.2.2.

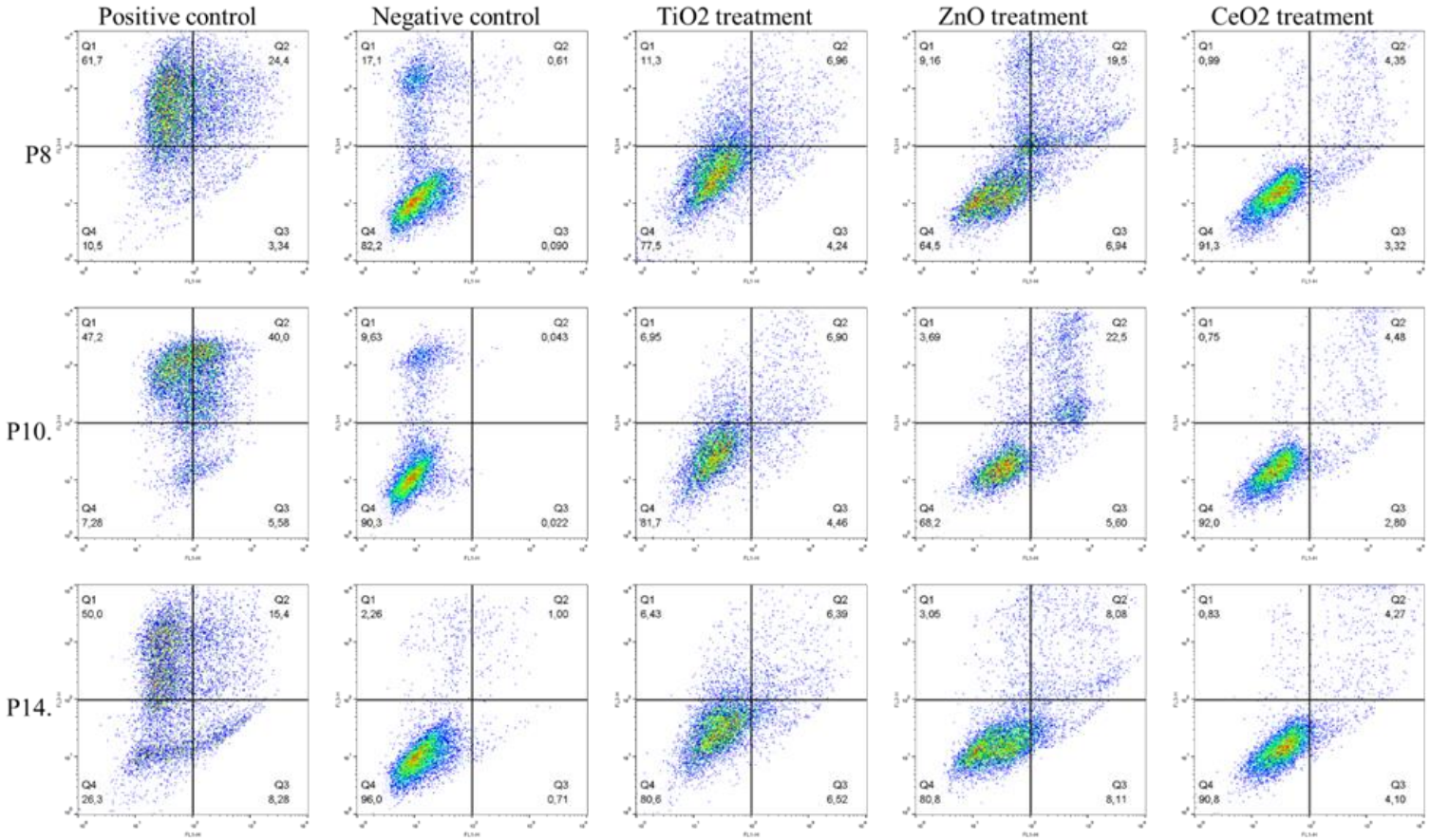

Figure 4.7: FACS readings of JEG-3 cells treated with annexin V (x-axis) and propidium iodide (y-axis) following nanoparticle (IC50) exposure.

Cells were exposed for 48 hours to nanoparticles at IC50 concentration and 24 hours to staurosporine for the positive control. Q1 = cell debris (dead cells), Q2 = late apoptosis, Q3 = Early apoptosis, Q4 = live cells. Percentage of Q4 (represented by the number in the corner of each quadrant) is used as percent viability of each control and treatment. TiO_2 = titanium dioxide, ZnO = zinc oxide, CeO_2 = cerium dioxide. As expected, positive control shows a very pronounced shift from Q4 to Q1, and when exposed to nanoparticles the cells shift to Q2 and Q3, indicating the cells undergo apoptosis in the presence of nanoparticles.

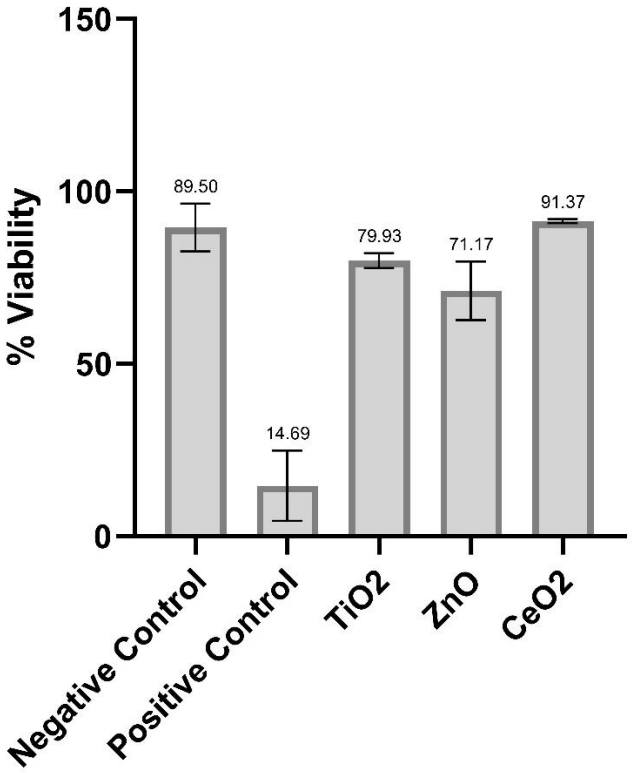

Figure 4.8: Mean of percent viability (viable cell proportion) of JEG-3 cells in FACS analysis following nanoparticle treatment at IC50 concentrations.

Negative control was untreated cells and positive control was cells exposed to Staurosporine. TiO_2 = titanium dioxide, ZnO = zinc oxide, CeO_2 = cerium dioxide. Numbers above bars represent the mean percent viability. N = 3

4.2.4 Effect of TiO_2, ZnO and CeO_2 on JEG-3 cell viability determined by immunocytochemistry (ICC)

As expected, for the negative control (untreated cells), we observed a uniform spread of nuclei with very few scattered and fragmented nuclei which indicates high viability (Figure 4.9 a). Also as expected, the opposite is observed for the positive control (Staurosporine treatment), where we observe a much-reduced number and a scattered distribution of nuclei, most of which are in the process of fragmenting indicating a high degree of cellular death and apoptosis (Figure 4.9 b). When exposed to nanoparticles at the same concentration as for flow cytometry (whether it is TiO_2, ZnO, or CeO_2), we observed the nuclei forming aggregates of fragmenting nuclei rather than having an even distribution of intact nuclei and an increased proportion of fragmented nuclei compared to the negative control in figure 4.9 a. (Figure 4.9 c,d,e). This indicates that the nanoparticles induce apoptosis and cell death. These results somewhat contradict the results obtained with flow cytometry especially for TiO_2 and CeO_2 because they did not show a significant

reduction in viability compared to the untreated cells (figure 4.8). However, we did observe a shift towards apoptosis when exposed to either TiO_2, ZnO, or CeO_2 as discussed in the section 4.2.3 which supports our observations here.

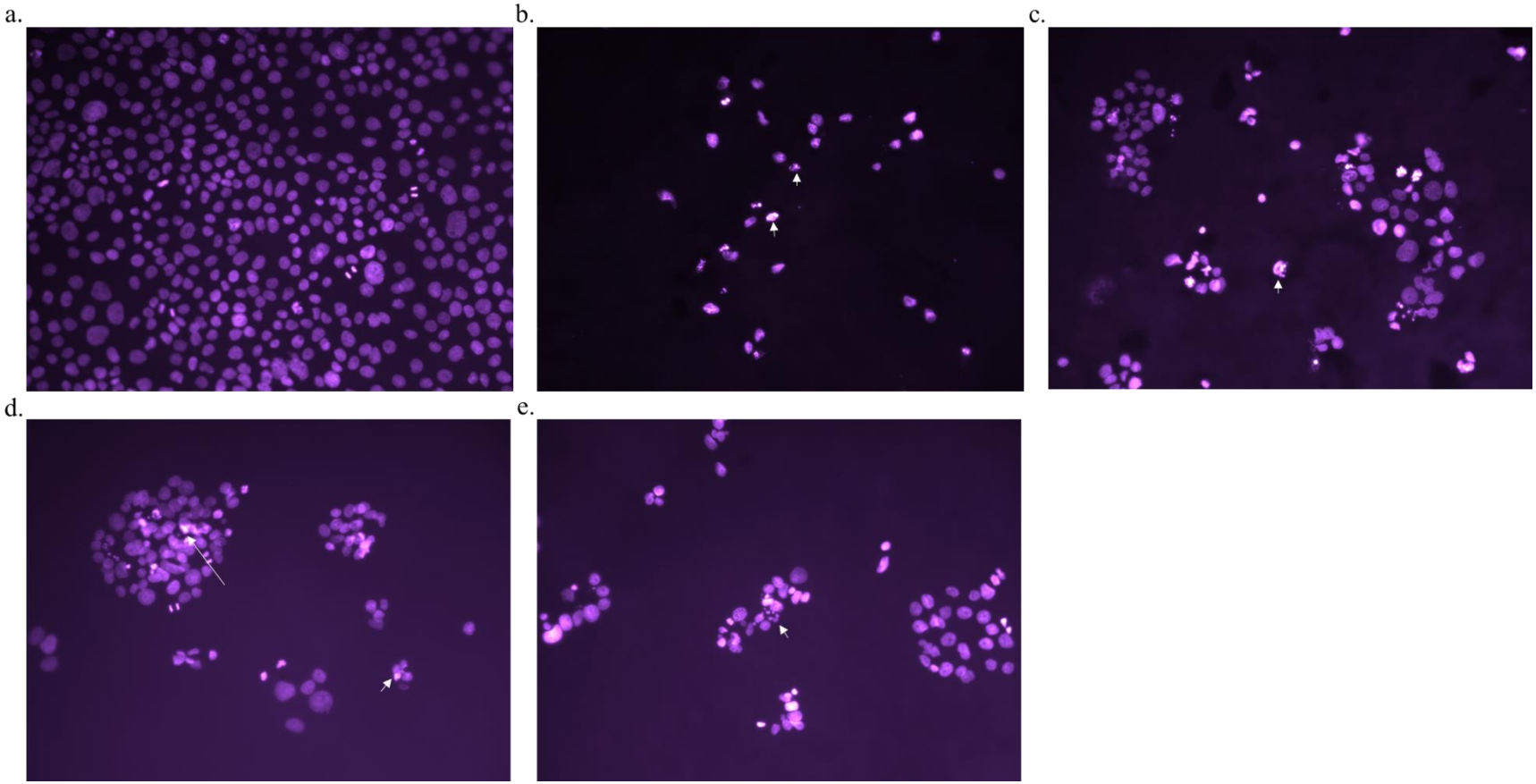

Figure 4.9: JEG-3 cell nuclei stained with Hoechst visualized at λex = 330nm – 380 nm.

Cells were visualized at 20X magnification with Nikon Eclipse E800 microscope (a) Negative control, (b) Positive control, (c) TiO_2 (titanium dioxide) exposure, (d) ZnO (zinc dioxide) exposure, (e) CeO_2 (cerium dioxide) exposure. Negative control consisted of untreated JEG-3 cells and positive control were cells treated with staurosporine for 24 hours. Untreated cells have a consistent and even distribution of intact nuclei. The positive control shows a drastically reduced number of nuclei most or which are fragmented or deformed. Exposure to nanoparticles led to an uneven distribution of nuclei and an increased proportion of fragmented or deformed nuclei. White arrows indicate fragmented nuclei. P = 20.

5 Discussion and Perspectives

Our wastewater data shows that, following the initial COVID-19 measures which involve a sudden reduction and perturbation in human activity due to confinement, there is a temporary drop in water contamination which goes in line with what was observed in previous literature (Najah, et al. 2021; Sarkar, et al. 2021) with regards to the effects of the COVID-19 pandemic on the quality of water.

Locally, this is further supported by the reduction in water contamination for DCO, DBO5C, and MES observed in 2017 and 2019 (figure 4.1) during times of flooding. We see the same trend again in 2020 between March and may when the COVID-19 measures were first put in place (figure 4.1). The same trend does not follow in 2020 and 2021 for new waves of COVID-19 (figure 3.2; figure 4.1 d) but following the first wave despite the alleviation of measures, human activity has only returned anywhere close to pre-COVID levels in post vaccination in 2021 with the introduction of the vaccine passport. The next lockdown occurred at the end of that year with the appearance of the OMICRON variant, but our data is limited to the end of 2021. These observations pertain to station Jean R Marcotte which applies to all of Montreal.

The strongest evidence we found that the reduction of human activity contributes to the reduction of wastewater contamination for DCO, DBO5C, and MES is our observations for station Ile Notre Dame. That station specifically serves its namesake, which is a strictly commercial area which hosts the Laronde amusement park. Areas such as this were kept closed since the beginning of the pandemic through 2021. As such, as seen in figure 4.3, wastewater contamination dropped drastically in with the beginning of the COVID-19 measures and has never recovered anywhere near to pre-pandemic levels. This strongly suggests a link between the COVID-19 measures and a reduction in environmental contamination. Interestingly, we observed the inverse for station Fabreville, where it is shown that once COVID-19 measures were being lifted across the province starting with vaccination around May 2021, water contamination levels actually shot up above pre-pandemic levels as seen in figure 4.4 and is further confirmed when we observed that overall contamination in terms of DCO, DBO5C and MES all roughly double in 2021 compared to previous years (figure 4.5). Station Fabreville serves, at least in part, the north of Quebec, so we propose this apparent discrepancy in data is a result of the tourism boom that took place in Quebec once the COVID-19 restrictions were lifted in late spring 2021 (Quebec-City, 2022). Despite that, we did not observe a statistically significant increase of water contamination in terms of DCO and DBO5C (figure 4.2) and no increase at all for MES contamination (figure 4.2). Despite

that, there is a yearly incremental increase of DBO5C from 2017 to 2021 but this goes beyond the scope of this study. To reiterate, our data supports the data found in other studies discussed in section 1.1.4 that the initial COVID-19 measures lead to at least a temporary increase in water quality, and that improvement can even last in areas where human activity remains minimal such as Ile-Notre-Dame. Despite our lack of data to support an increase in water contamination as the pandemic progressed in Montreal, a lot of literature supports the fact that there was an increase in human exposure to endocrine disrupting chemicals during the pandemic related to the increase of sanitation and cleaning products and for nanoparticles being an emerging endocrine disruptor (Vayisoglu, et al. 2021; Gharpure, et al. 2020; Gupta, et al. 2018; Onyeaka, et al. 2022, Radwan, et al. 2021). MES serves as a good marker for general pollution and refers mostly to organic waste that's in the water, such as algae, bacteria, fecal matter but even sediments such as clay, sand, and rocks (University of Wisconsin-Madison. 2023) and is worth investigating when trying to understand how human activity in general contributes to water pollution. DCO specifically refers to oxidative species which is directly related to chemicals such as bisphenol A and Parabens which could seep into municipal waters (Meli, et al 2020; Samarasinghe, et al. 2018). Despite the increase in 2021 not being statistically significant there is still an observed increase compared to the previous years (figure 4.2 a). It is worth investigating further (data for 2022, 2023, and beyond) to see whether there is an even further increase in contamination which might establish a trend and show a significant increase down the line. Regardless it is worth investigating the effects of endocrine disruptors related to the effects the pandemic had on human behaviour (increased plastic and sanitation product use) and their potential effects on human health.

TiO_2, ZnO, and CeO_2 all have potential as oxidative agents. TiO_2 has known strong photooxidative properties which are exploited to make "self cleaning" surfaces (Fujishima, et al. 2000; Denning, et al. 2009). ZnO is specifically studied for applications of its capacity to photo-oxidize carbon monoxide into carbon dioxide (Luo, et al. 2020), although its capacity to react with other organic compounds is not well known, it is capable of oxidative reaction with organic compounds (Kolodziejczak-Radzimska, et al. 2014). Lastly, CeO_2 is known to be capable of photooxidation of organic compounds and is used in a variety of applications in many industries for these properties (Xu, et al. 2014; Ma, et al. 2018). That makes the three nanoparticles potential pollutants whose increase in usage during the COVID-19 pandemic and exposure could have contributed to the increase in the presence of oxidative species in municipal wastewater, especially relevant in the context of chemical oxygen demand (referred to as DCO) which measures oxygen consumed by chemical reaction as a total rather than due to biochemical oxidation alone as measured by biochemical oxygen demand (referred to as DBO5C). Thus, we study in the second part of this

project the effect of these three nanoparticles on the placental using JEG-3 cells as a model for cytotrophoblasts.

Our results show that all three nanoparticles reduce the viability of JEG-3 cells in the context of mitochondrial activity (the nature of MTT assay) and induce apoptosis in the cells (figure 4.6; figure 4.7, figure 4.9). However, we observed a discrepancy in our results when looking at the viability data of the flow cytometry assay where we observed that TiO_2, and CeO_2 did lead to a significant reduction in viability of JEG-3 cells compared to untreated JEG-3 cells and ZnO did not have a 50% reduction in viability as expected, although it is significant (figure 4.8).

This discrepancy could be explained by the difference between the mechanism analyzed by the MTT assay compared to flow cytometry of ICC. The MTT assay relies on an indirect method to measure cellular viability, which is the activity of the mitochondrial dehydrogenase and oxidoreductase activity compared to FACS flow cytometry and ICC which measures apoptotic and dead cell count and images cells and their constituent parts respectively. As such cellular viability is inferred indirectly from the activity of the mitochondria which can be reflective of cellular viability, and it has been shown that other variables might affect cellular viability, MTT formation, and mitochondrial viability (Ghasemi, et al. 2021). A study done on rat neurons showed that while MTT was reduced by isolated mitochondria when exposing MTT to intact cells, MTT reduction was mainly confined to intracellular vesicles supported by the fact that MTT was membrane impermeable and MTT accumulated within endosomal and lysosomal vesicles. (Liu, et al. 1997). In addition, there are studies that suggest that NADH dehydrogenase and oxidoreductase are involved in caspase-independent apoptosis which is primarily mediated by mitochondrial pro-apoptotic molecules. (Wang, et al 2016; Negara, et al 2020). They suggest that mitochondrial NADH dehydrogenase Nde1 can potentially act as an apoptotic inducing factor (AIF) signalling molecule (Herrmann, et al. 2020). Otherwise, the AIF contains flavin adenine dinucleotides (FAD) and nicotinamide adenine dinucleotide (NAD) prosthetic groups which are conserved NADH oxidoreductases (Daugas, et al. 2000). In addition, studies show that both ZnO and TiO_2 have the capacity to induce apoptosis in different types of cancer cells (JEG-3 cells are derived from choriocarcinoma), although, through caspase dependent pathways (Akhtar, et al. 2012; Yoo, et al. 2012; Kim, et al. 2019). It has been shown that all three nanoparticles tested in our study lead to placental growth restriction and fetal growth restriction, as well as interfere with the trophoblast cell proliferation (Chen, et al. 2020; Zhang, et al 2018; Nedder, et al. 2020). Moreover, there is evidence to suggest fetal growth restriction is caused to some degree by the increased apoptosis of trophoblast cells (Murthi, et al. 2005, Crocker, et al 2003).

Thus, our results suggest it is likely that the nanoparticles tested in our study can induce apoptosis and cellular death in JEG-3 due to our observations of an increased instance of apoptosis (figure 4.7, figure 4.9), and it is unclear how their effects on cellular viability might skew the results of the MTT assay, and some studies do show that the nanoparticles in question have a direct effect on mitochondrial function (Anand, et al. 2023; Nalika, et al. 2015; Ma, et al. 2021) which when taken into consideration with the complicated nature of apoptosis and mitochondrial dehydrogenase and the potential interaction the nanoparticles could have with the enzyme, could lead to potential increased deficiency in the production of formazan which could lead to a lower viability reading. We would need to investigate further into the mechanism and causes of cellular death and decreased viability to fully understand the apparent discrepancies in our studies, such as the measurement of oxidative species, determine which apoptotic pathway the cells go through, checking for other forms of cellular death such as autophagy, which one study shows titanium dioxide induces autophagy in trophoblast cells (Zhang, et al. 2018).

It is also important to note that the JEG-3 cells are a secondary cell line model, and they are derived from choriocarcinoma cancer cells, but considering the effect of the nanoparticles on them it is highly plausible that using a primary culture trophoblast cell will produce similar results based on the literature. For example, two studies demonstrated that CeO_2 is easily up taken by primary cytotrophoblast cells but also induces caspase mediated apoptosis and decreases CT differentiation and fusion as well as important pregnancy hormone secretions such as hCG, and hPL (Deval, et al. 2023; Nedder, et al. 2020). While we did not find any literature about the impact of TiO_2 and ZnO on primary trophoblasts, as mentioned in the section 1.3.5 it has been shown in both vivo models of mice as well as in other trophoblast cells line such as HTR-8/SVneo cells that ZnO can induce placental stress as well as apoptosis of trophoblast cells in mice and TiO_2 induces apoptosis in mice placenta impeding its growth as well as autophagy of HTR-8/SVneo which could be worth investigating in a further study for JEG-3 cells and primary cultures (Chen, et al. 2020; Zhang, et al. 2018; Zhang, et al. 2017).

The nanoparticles analyzed also serve as potential therapeutic agents against cancer cells, which can explain our data on JEG-3 cell viability. Several studies discuss the potential of TiO_2, ZnO, and CeO_2 in cancer treatment, where they show that all three nanoparticles have the capacity to selectively induce apoptosis in tumoral cells while having a relatively small effect on the respective healthy tissue (Anjum, et al. 2021; Li, et al. 2020; Datta, et al. 2020). Since JEG-3 are derived from cancer cells, the nanoparticles might have a stronger detrimental effect on their viability than they would on primary trophoblasts. So, while the literature generally seems to support that the

nanoparticles would be harmful to primary trophoblasts, the detrimental effect on viability might be more potent on cancers derived from trophoblast cells, which opens the door for potential cancer treatments in certain doses.

Moreover, when taken into account our results on JEG-3 cells which serve as a model for trophoblast cells in early pregnancy, which showed us that exposure to all three nanoparticles lead to some degree of reduced cellular viability as well as a shift towards apoptosis with ZnO having the most pronounced and consistent effect, the wastewater data and the literature all supporting an overall increase of oxidative pollutants in municipal wastewater as well as general increase in exposure to those nanoparticles due to human behaviour changes, it is plausible that the pandemic lead to an overall increase in exposure to those three nanoparticles which in turn could lead to an increase in pregnancy-related pathologies related to growth restriction of the placenta and reduced differentiation of trophoblast cells. However, this remains to be studied. Moreover, due to the nature of nanoparticles, these effects might take more than a single generation to manifest, since the toxic effects of nanoparticles as endocrine disruptors are not only in their direct effect on initial exposure, but the fact that they are very prone to bioaccumulation which has cross-generational effects (Kuehr, et al. 2021; Drobna, et al. 2017). The principles of developmental origin of health and diseases (DoHaD) suggest that EDCs can have long-term effect on human health (Lacagnina, et al. 2019). Their impact is most severe on stages of human development which involve major changes to the organism and rapid cellular proliferation, making embryonic and fetal development a very vulnerable life state to such compounds (Lacagnina, et al. 2019; Diamanti-Kandarakis, et al. 2009). Similarly, to how various EDCs lead to the precocious puberty of children in some parts of the world, and it is unknown when initial exposure was made, it is likely that those toxins were passed from parents to children and already had an effect since conception, which leads to a disturbance in their endocrine system (Ozen, et al. 2011; Papadimitriou, et al. 2021). In the same way that the effects of compounds such as BPA have been extensively studied for decades, there need to be given equal attention to compounds such as the three nanoparticles investigated in this study. TiO_2, ZnO, and CeO_2 have a clear effect on JEG-3 cells, which act as a model for trophoblast cells, but further studies should be conducted with BeWo cells to give a more robust image of their effect on trophoblast cell models, and ideally on primary culture trophoblast cells harvested from an actual placenta. As for the wastewater data, the data should also be investigated for 2022 and 2023 to see how water contamination changed with the gradual full relaxation of COVID-19 restrictions in Quebec and ultimately the return to pre-COVID-19 habits and regulations.

6 Conclusion and recommandations

Our study shows that while COVID-19 pandemic sanitary measures had positive impacts such as temporary increase of wastewater quality during the initial phase of the lockdowns expressed by the decrease of water contamination in the initial lockdown of the pandemic on March 13 as shown in the figure 4.1 and further supported by figure 4.3, but despite that based on the literature, it seems that overall, the change in human habits, as well as COVID-19 regulations which encouraged the use of single-use plastics, increased sanitation product use led to the overall increased exposure of potential nanoparticles (such as TiO_2, ZnO, and CeO_2) and contaminants, indicated by the overall rise in water contamination in oxidative species, and is further supported by existing literature, which can lead to a new set or increased health problems. Considering our results with regards to the effects of the TiO_2, ZnO, and CeO_2 on JEG-3 cell viability and increased apoptosis, it is clear these nanoparticles have a detrimental effect on their viability and induce their apoptosis of with the stronger effect observed with ZnO with a IC50 value of only 146.5 mg/ml compared to 836.3 mg/ml for TiO_2 and 772.2 mg/ml for CeO_2 at 48 hours of exposure. Moreover, in the context of increased exposure to endocrine disrupting chemicals, the nanoparticles are likely to have a detrimental effect on placental development and function, which in turn can have detrimental effects on embryonic and fetal development, which could have a life-lasting impact on the quality of life of many potential children and people.

We recommend that further studies should be done into the possible effects on viability that the nanoparticles might impose on related cell lines such as BeWo and JAR to obtain a more comprehensive picture of the effects nanoparticles have on choriocarcinoma cell lines. Furthermore, additional effects on viability by the nanoparticles should be investigated such as, increase of reactive oxidative species, the effects on other cellular structures such as the cytoskeleton, the cellular membrane, and the mitochondria, and ideally, repeat the experiment on primary trophoblast cell culture from placental explants which would allow to obtain a clearer and more reliable picture on the effects of the nanoparticles on the placenta.

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8 ANNEXE I

8.1 Cytospin Microscopy

8.1.1 Materials and methods

The cells were seeded in a 12-well plate at $1.0x10^5$ cells per well, 0.5 ml culture per well. The cells were incubated and treated similarly to flow cytometry (section 3.2.5) with three biological replicates. Cells were split then spun in a Sakura Cyto-Tek 4325 centrifuge (Torrence, California) at 1000g for 5 minutes. Then the cells on the slides were fixed with Hema 3 Fixative, washed, stained with Hema 3 Solution I dye, washed, stained with Hema 3 solution II dye obtained from Fisher Scientific (Waltham, MA) and washed. Positive control was the cells being treated with 1 µM Strausporine, and negative control was untreated cells. The cells were then observed under a Leica Microsystems DM IL LED microscope (Wetzlar, Germany) at 20X magnification.

8.1.2 Results

Cytospin did not produce conclusive results. We can see that untreated cells (figure 8.1 a) resulted in a mostly intact cells, and the positive control (staurosporine treatment) led to cellular death (figure 8.1 b). Unfortunately, the dye appears to form patches around the cells when treated with TiO_2 (figure 8.1 c). Cells treated with ZnO and CeO_2 produced pictures where it is hard to distinguish individual cells let alone their nuclei (figure 8.1 d,e).

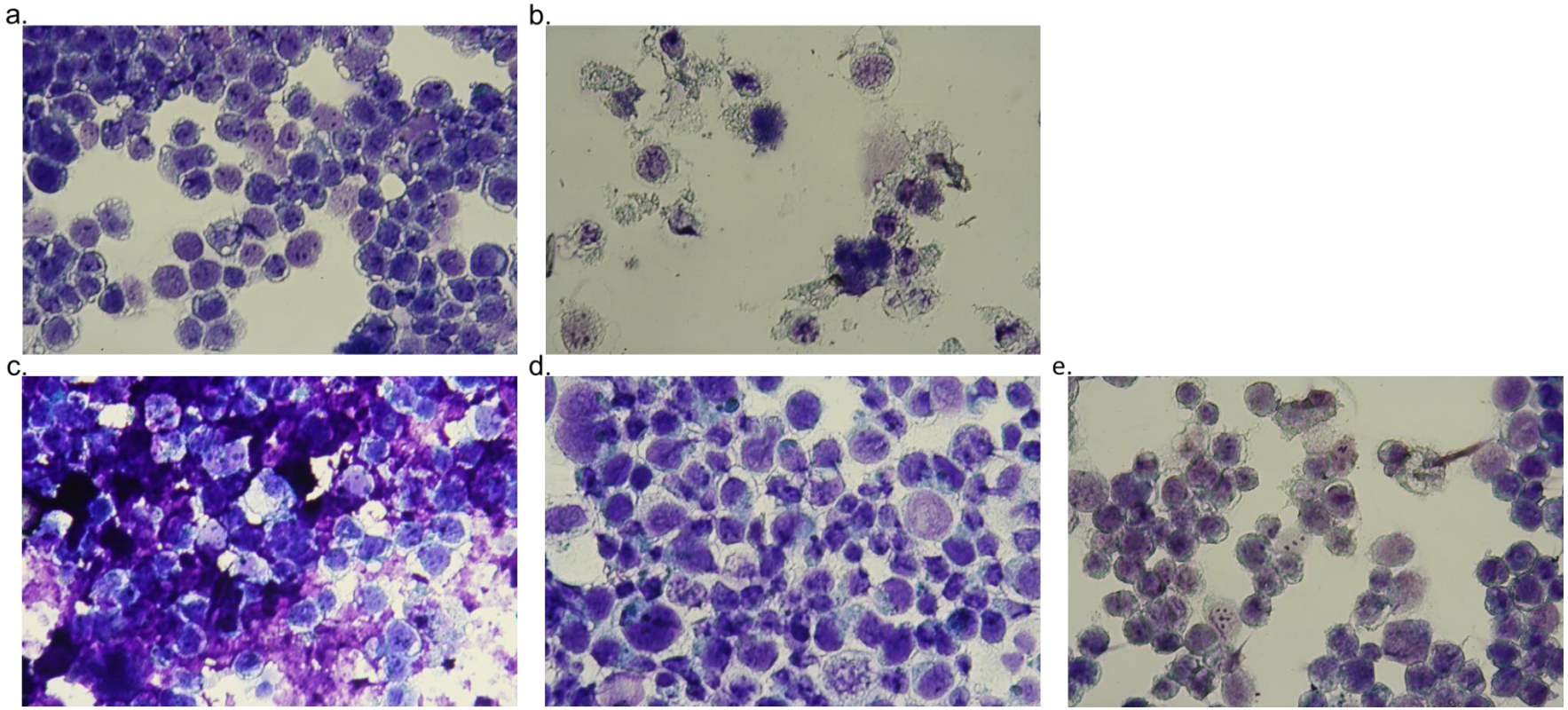

Figure 8.1: Cytospin staining of JEG-3 cells. Cells we stained with Hema 3 solution I and Hema 3 solution II and viewed at 20x magnification. (a) negative control (untreated cells), (b) positive control (staurosporine treatment), (c) titanium dioxide (TiO_2) treatment, (d) zinc oxide (ZnO) treatment, (e) cerium dioxide CeO_2 treatment. P = 20.

8.2 Nanoparticle characterization

a.

Sample Name:	TiO2 - Media 5		
SOP Name:	mansettings.nano		
File Name:	NPs media test.dts	Dispersant Name:	Water
Record Number:	40	Dispersant RI:	1.330
Material RI:	1.59	Viscosity (cP):	0.8872
Material Absorbtion:	0.010	Measurement Date and Time:	March-03-22 2:09:25 PM
Temperature (°C):	25.0	Duration Used (s):	60
Count Rate (kcps):	298.9	Measurement Position (mm):	4.65
Cell Description:	Disposable sizing cuvette	Attenuator:	8

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	45.97	Peak 1:	422.7	58.6	291.0
PdI:	1.000	Peak 2:	32.84	19.4	14.70
Intercept:	0.935	Peak 3:	9.642	18.7	3.238

b.

Sample Name:	ZnO - MQ 5		
SOP Name:	mansettings.nano		
File Name:	NPs media test.dts	Dispersant Name:	Water
Record Number:	15	Dispersant RI:	1.330
Material RI:	1.59	Viscosity (cP):	0.8872
Material Absorbtion:	0.010	Measurement Date and Time:	March-03-22 12:41:06 PM
Temperature (°C):	25.0	Duration Used (s):	160
Count Rate (kcps):	28.0	Measurement Position (mm):	4.65
Cell Description:	Disposable sizing cuvette	Attenuator:	11

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	134.6	Peak 1:	14.04	59.7	7.288
PdI:	0.176	Peak 2:	81.99	17.6	33.47
Intercept:	0.251	Peak 3:	0.8415	12.0	0.1971

c.

Sample Name:	CeO - Media 5		
SOP Name:	mansettings.nano		
File Name:	NPs media test.dts	Dispersant Name:	Water
Record Number:	30	Dispersant RI:	1.330
Material RI:	1.59	Viscosity (cP):	0.8872
Material Absorbtion:	0.010	Measurement Date and Time:	March-03-22 1:35:54 PM
Temperature (°C):	25.0	Duration Used (s):	60
Count Rate (kcps):	293.9	Measurement Position (mm):	4.65
Cell Description:	Disposable sizing cuvette	Attenuator:	9

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	19.57	Peak 1:	13.66	51.3	6.783
PdI:	0.471	Peak 2:	89.05	48.7	64.08
Intercept:	0.924	Peak 3:	0.000	0.0	0.000

Figure 8.2 Dynamic light scattering test of nanoparticles in EMEM media. (a) Titanium dioxide (TiO_2) has aggregates of 9.743 nm, 32.84 nm, and 422.7 nm. (b) Zinc oxide (ZnO) has aggregates of 0.8415 nm, 81.99 nm, and 14.04 nm. (c) Cerium dioxide (CeO_2) has aggregates of 89.05 nm, and 13.66 NM.

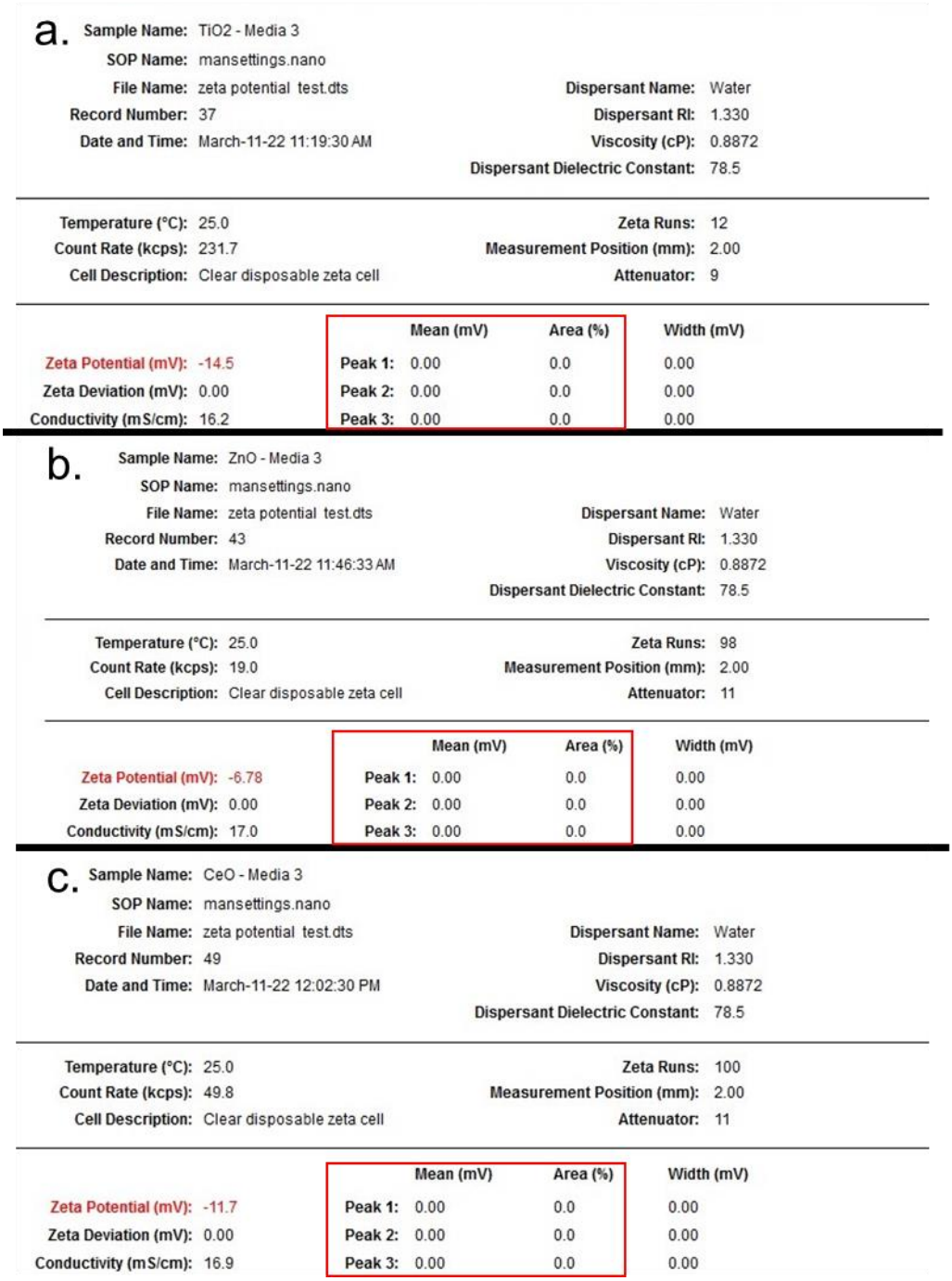

Figure 8.3: Zeta potential test of nanoparticles in EMEM media. All three nanoparticles show completely neutral charge when in media.