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In vitro evaluation of the effect of oxidative stress in placental function

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IN VITRO EVALUATION OF THE EFFECT OF OXIDATIVE STRESS IN PLACENTAL FUNCTION

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituo de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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If there are people who believe in you,
even if it's just one, just do it.
Ko Shinwon - PENTAGON

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Resumo

A placenta é um órgão temporário, mas essencial, que estabelece um ponto de ligação entre a mãe e o feto. As suas funções incluem não só o fornecimento de oxigénio e nutrientes ao embrião durante o seu desenvolvimento, como também a produção de hormonas e enzimas e a proteção do feto. O aumento da concentração de espécies reativas de oxigénio numa placenta saudável é algo natural devido à sua alta atividade mitocondrial, mas sabe-se que este aumento nos níveis de espécies reativas de oxigénio é acompanhado por um aumento nos níveis de antioxidantes que protegem a placenta. Quando este balanço é quebrado, dizemos que estamos perante uma situação de stress oxidativo. O aumento dos níveis de stress oxidativo na placenta está associado com o surgimento e desenvolvimento de várias patologias da gravidez, tais como a pré-eclâmpsia e a diabetes mellitus gestacional, e com o surgimento de doenças no feto em fases posteriores ao seu nascimento. Para além disso, as grávidas são expostas a inúmeros contaminantes durante o período de gestação. As formas mais comuns de exposição incluem fármacos, tabaco, álcool, pesticidas e metais pesados. Estes tóxicos, em contacto com a placenta, podem contribuir para a desregulação da sua atividade e podem também interferir com as fases iniciais da placentação. Muitos xenobióticos, tais como o álcool, pesticidas e o cádmio, causam um aumento dos níveis de stress oxidativo.

Com o nosso estudo, pretendemos entender como é que os trofoblastos extravilositários , que são as principais células envolvidas no processo de placentação, são afetadas por níveis altos de stress oxidativo. Para isso expusemos células HTR8/SVneo a *tert*-butilhidroperoxido (0.5 μM; 24h), que foi capaz de aumentar os níveis de stress oxidativo, avaliado com a quantificação da peroxidação lipídica e dos níveis de carbonilação proteica. Em condições de aumento de stress oxidativo, verificamos um decrescimento das taxas de proliferação das células HTR8/SVneo, na sua capacidade migratória e um aumento no seu índice de apoptose, embora não tenha havido interferência com a taxa de necrose e com a captação de glucose pelas células. Os nossos resultados demonstram que níveis elevados de stress oxidativo interferem com características essenciais dos trofoblastos extravilositários para o processo de placentação (proliferação, migração e apoptose). Este efeito direto do stress oxidativo nos trofoblastos extravilositários pode contribuir para a associação que se verifica entre stress oxidativo e várias patologias da gravidez e pode também contribuir para os efeitos deletérios de contaminantes indutores de stress oxidativo na gravidez.

Abstract

The human placenta is a temporary, yet essential, organ that connects the mother with the foetus. Its roles include providing oxygen and nutrients to the embryo during its development, but it is also important in producing hormones and enzymes and in protecting the foetus. The increase of reactive oxygen species concentration is natural in a healthy placenta due its high mitochondrial activity and is followed by a parallel increase in antioxidants. When this balance is broken, we are facing a situation of oxidative stress. Increased placental oxidative stress levels are associated with the onset and progression of several pregnancy disorders, including preecampsia and gestational diabetes mellitus, and with programming the foetus for metabolic diseases later in life. Moreover, pregnant women are exposed to numerous xenobiotics during the gestation time. The most common ways of exposure include medicinal drugs, smoke, alcohol, pesticides or heavy metals. These toxics, when in contact with the placenta, can compromise their main functions and the early stages of placentation as well. Several xenobiotics (including alcohol, pesticides and cadmium) increase placental oxidative stress levels.

With our study, we aimed to understand how extravillous trophoblasts, which are the main cell type involved in the placentation process, are affected by oxidative stress conditions. For this, we exposed HTR8/SVneo cells to *tert*-butylhydroperoxide (0.5 μM; 24h), which was able to increase oxidative stress levels, as evaluated by quantification of lipid peroxidation and protein carbonyl levels. These conditions of increased oxidative stress levels were found to decrease the proliferation rates of HTR8/SVneo cells, their migratory capacity and to increase apoptosis index, although it did not interfere with necrosis and with glucose cellular uptake. Our results thus show that oxidative stress interferes with extravillous trophoblast characteristics essential for the placentation process. This direct effect of oxidative stress on extravillous trophoblasts may contribute to the association between oxidative stress and pregnancy disorders and may also contribute to the deleterious effects of oxidative stress-inducing xenobiotics to the foetus.

Index

Figure Index

Abbreviations Index

$^{\bullet}$HO	Hydroxyl radical
^{3}H-DG	^{3}H-deoxy-D-glucose
AB-AM	Antibiotic/ antimycotic solution
$CaCl_2$	Calcium chloride
CAT	Catalase
cm^2	Square meter
CO_2	Carbon dioxide
DAPI	4,6 Diamino-2-phenylindole
DNA	Deoxyribonucleic acid
DNP	Protein carbonyls producing hydrazones
DNPH	2,4-Dinitrophenylhydrazine
dUTPs	deoxyribonucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
eEVT	Endovascular extravillus trophoblasts
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GLUT1	Glucose transporter 1
GPx	Glutathione peroxidase
h	Hours
H_2O	Water
H_2O_2	Non-radical hydrogen peroxide
HCL	Hydrochloride
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
iEVT	Interstitial extravillousl trophoblasts
KCl	Potassium chloride
LDH	Lactate dehydrogenase
M	Molar mass
m/v	Mass concentration
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
min	Minutes
mm	Millimeter
NaCl	Sodium chloride
NAD(P)H	Dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nM	Nanomolar
$O_2^{\bullet-}$	Superoxide anion radical
ºC	Celsius degree

PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
rpm	Revolutions per minute
SOD	Superoxide dismutase
SRB	Sulforhodamine B
STB	Syncytiotrophoblast
TBA	2-thiobarbituric acid
TBH	*tert*-butylhydroperoxide
TCA	Trichloroacetic acid sodium salt
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferasedUTP-mediated nick end labeling
v/v	Volume fraction
µL	Microliter
µM	Micrometre

Introdução

1. Oxidative stress

Oxidative stress is the term used when the balance between reactive oxygen species (ROS) formation and their clearance by defensive antioxidants in the cell is broken, favoring the formation of ROS (Aouache et al., 2018; Pereira & Martel, 2014; Wu et al., 2015). This imbalance leads to a disruption of the redox control and to molecular damage (Aouache et al., 2018).

ROS include free-radical intermediates of cell reactions that involve oxygen, the most common being the superoxide anion radical ($O_2^{\bullet -}$), the hydroxyl radical ($^{\bullet}HO$) and the non-radical hydrogen peroxide (H_2O_2) (Pereira & Martel, 2014; Wu et al., 2015). The existence of one, or more, unpaired electrons is what gives these compounds a high instability (Pereira & Martel, 2014), causing physiological and structural damage to the cell membrane, proteins, lipids and DNA chains (Aouache et al., 2018).

Besides being a product of aerobic processes, ROS can also be generated from other mechanisms such as xenobiotics and drug metabolism, radiation exposure, monoamine oxidase activity (this enzyme deaminates biogenic amines in the outer membrane of the mitochondria producing H_2O_2), protein folding occurring in the endoplasmic reticulum (large production of $O_2^{\bullet -}$ anion radicals), inflammatory responses (high production of H_2O_2 and $O_2^{\bullet -}$) and reactions catalyzed by dinucleotide phosphate (NAD(P)H) oxidase (Pereira & Martel, 2014).

Low antioxidant concentrations in the cell can also result in oxidative stress. These antioxidants can be enzymatic (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) or non-enzymatic (vitamin C and E, nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH)). Their functions include not only the degradation of ROS or their transformation in less reactive products, but also the maintenance of the agents involved in the antioxidant reactions (Aouache et al., 2018).

2. The Placenta

The placenta is a temporary yet essential organ that is formed during the implementation phase of the embryo (Staud & Karahoda, 2018; Wong & Cox, 2017). It connects the mother with the embryo and plays multiple roles during pregnancy, the most

important being providing oxygen and nutrients to the embryo (Gude et al., 2004; Syme et al., 2004).

Placental functions can be categorized as transport and metabolism, protection of the foetus and endocrine functions. This organ provides oxygen, nutrients, water (Gude et al., 2004), electrolytes, steroid and hormones, growth and regulatory factors (for example, acetylcholine, prostaglandins and insulin like growth factors) (Gupta & Gupta, 2017) while taking care of waste products (including CO_2) of the foetus (Gude et al., 2004). The placenta has also a main role in providing a flow of chemical information between the mother and the foetus that is essential for homeostasis during all stages of embryogenesis (Gupta & Gupta, 2017). Besides working as a link between two distinct individuals, the placenta also acts as a barrier protecting the embryo from xenobiotics, infections and pathogenic agents that may be present in the maternal blood (Syme et al., 2004). While acting as a barrier, some of the antibodies present in the mother bloodstream can be transferred to the foetus by the placenta (Gupta & Gupta, 2017).

2.1. Placentation

Human fertilization, that occurs on the fallopian tromps, originates a single cell from two haploid cells with completely different genome called zygote. This cell undergoes multiple mitosis until it reaches an early stage of undifferentiated cells called morula. Meanwhile it initiates its journey to the uterus cavity where placentation occurs. The trophoblast is the first cell lineage to differentiate and, when the trophoblasts are well stablished, the blastocyst differentiates into embryoblast and blastocoele (or blastocyst cavity). The human placenta will develop from the trophoblast cells, while the inner part of the blastocyst will originate the embryo and umbilical cord (embryoblast) and the placental mesenchyme (blastocoele) (Huppertz et al., 2006; Turco & Moffett, 2019).

From the morphological point of view, the human placenta is of hemochorial type, which means that, in humans, the fetal tissue is in direct contact with the maternal blood (Gupta & Gupta, 2017). This point of contact is created by the time the trophoblast starts invading the endometrium, rapidly proliferating and differentiating, allowing the implantation of the blastocyst. The outer layer differentiates into syncytiotrophoblast (STB), that acts to protect the fetus from the maternal immune response and as a nutrient and metabolite exchange membrane, while the inner cluster differentiates into extravillous trophoblast (EVT) that migrate into the maternal decidualized endometrium and act on the spiral arteries in order to provide oxygen and nutrients to the placenta and the fetus (Forbes & Westwood, 2010; Huppertz et al., 2006; Wu et al., 2015).

The STBs are the differentiated trophoblasts responsible for synthesizing proteins (human chorionic gonadotropin and somatomammotropin, growth hormone, adipokines) and steroid hormones (progesterone, oestrogens) to the placenta. They are also the major unit of the placental barrier, mediating the nutrient transportation between mother and foetus, and are responsible for the organization and architecture of the placenta (Staud & Karahoda, 2018).

On the other hand, the EVTs are responsible for the remodeling of the spiral arteries as they invade the decidual arteries of the mother. They provide low-pressure, high blood flow to the foetus (Staud & Karahoda, 2018). EVTs also invade into uterine glands, connecting all the luminal structures with the intervillous space and providing nutrients to the embryo with the secretions from these glands during the first trimester of pregnancy (Moser & Huppertz, 2017). There are two different phenotypes of EVTs cells: the interstitial trophoblasts (iEVT), differentiated cells that detach from the placental villi and invade the decidua, and the endovascular trophoblasts (eEVT) that participate in the remodeling of the maternal spiral arteries (Staud & Karahoda, 2018). The Figure 1 shows the different types of trophoblast in the early stage of placentation.

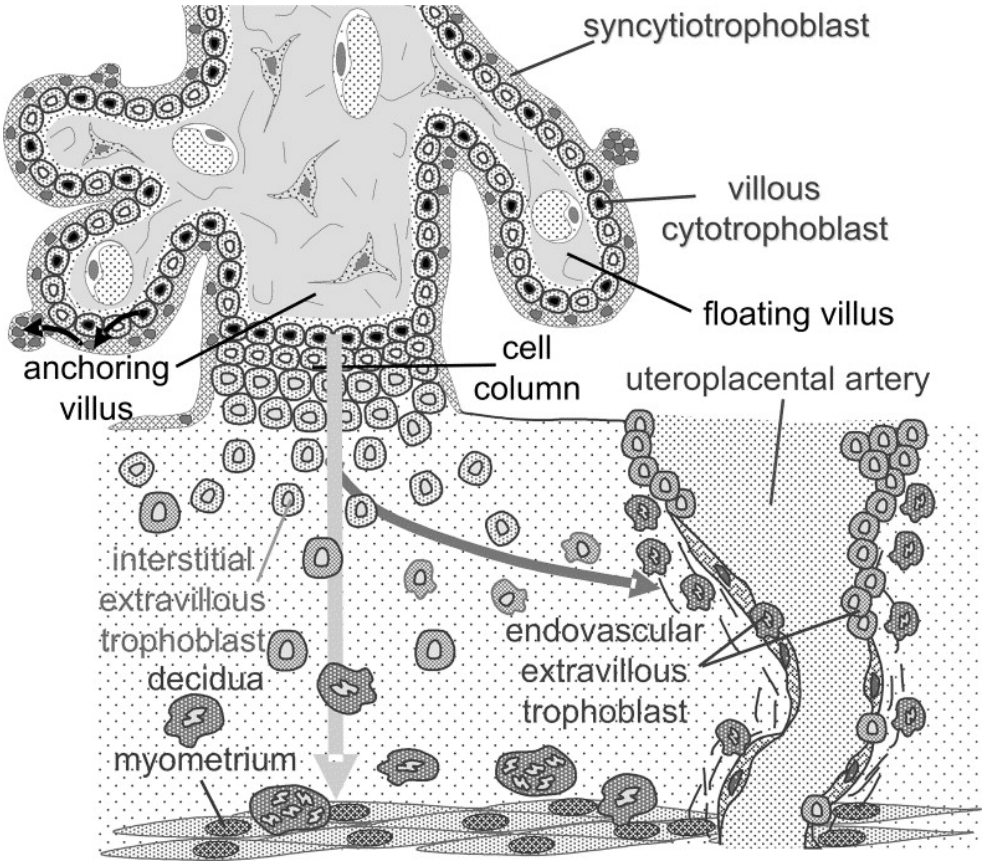

***Figure 1**: Different type of trophoblasts in the early stage of placentation. Taken from: Apoptosis and its role in the trophoblast. by Huppertz et al., 2006, American Journal of Obstetrics and Gynecology, 195(1), 29–39*

The first step of the blastocyst implantation, when the cell attaches to the endometrium and starts invading, is denominated apposition and its followed by the adhesion phase, where a more stable attachment is stablished, and finishes with the proper cell invasion of the luminal epithelial cells (James et al., 2012) (Figure 2). The implantation occurs 6~7 days after the fertilization and the STBs are the main cells during this process since they produce the enzymes that digest the endometrium cells and allow the penetration of the trophoblast (Huppertz et al., 2006; Staud & Karahoda, 2018).

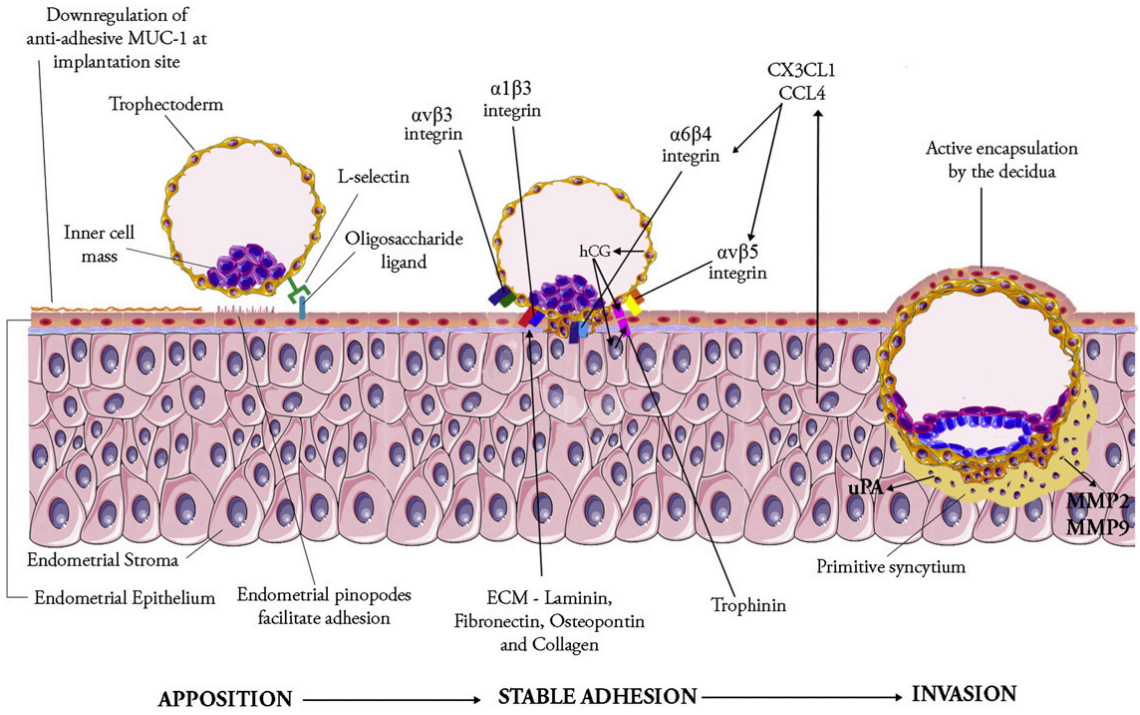

Figure 2: *The three stages of the blastocyst implantation. Taken from: Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? By James et al., 2012, Placenta, 33(5), 327–334*

An adequate trophoblast invasion and blastocyst implantation are essential for a proper formation and development of the placenta in order to provide blood, oxygen and nutrients to the foetus during pregnancy (Moser et al., 2018; Staud & Karahoda, 2018; Windsperger et al., 2017). In his context, unsuccessful trophoblast differentiation and function can lead to several complications of pregnancy including preeclampsia or malformation, cause fetal (mis)programming or even cause health problems in later life stages of the foetus (hypertension, depression, autism spectrum disorders, diabetes mellitus) (Staud & Karahoda, 2018). Although alterations in trophoblast invasion have been associated with adverse pregnancy outcome, little is known about how this alterations affects the placentation and the normal functioning of the placenta (Moser et al., 2018; Windsperger et al., 2017).

3. Oxidative stress in the placenta

High levels of oxidative stress are expected to occur in the placenta, which is an organ with a high mitochondrial activity. However, this increase in ROS production is accompanied by an increase in placental antioxidation protection, especially with the activity of some of the enzymes mentioned above (Pereira & Martel, 2014). Indeed, the onset of maternal blood circulation in the placenta is associated with a raise of oxidative stress levels in the trophoblast, and this oxidative stress rise is followed by an increase of glutathione peroxidase and catalase concentrations. The increase of ROS in the placenta also contributes to trophoblast invasion and is also involved in cellular signaling pathways that contribute to the normal development and cell function (Schoots et al., 2018).

However, if the dynamic balance between the ROS generation and the antioxidants activity in the placenta is lost, it can result in some significant changes in the trophoblast morphology (Wu et al., 2015) and in several pregnancy complications that affect both the mother and foetus (Pereira & Martel, 2014; Schoots et al., 2018; Wu et al., 2015). Oxidative stress can also induce apoptosis, a homeostatic process in trophoblast cells that can also lead to autophagy in these cells. When these events occur in the cell in an unnecessary and dysfunctional way it can lead to placental pathologies (Wu et al., 2015) and fertility related diseases (Pereira & Martel, 2014). Some of the most well-known pregnancy complications related to high oxidative stress levels can include gestational diabetes mellitus, intrauterine growth restriction, spontaneous abortion and recurrent pregnancy loss, preeclampsia (Pereira & Martel, 2014). These will be outlined next.

3.1. Oxidative stress and pregnancy disorders

Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) occurs when a healthy pregnant woman develops glucose intolerance around the second half of gestation. This condition increases the chances of fetal macrosomia, perinatal mortality, and long-term risk of developing type 2 diabetes mellitus for the mother (that's why is considered a pre-diabetic state) and the fetus. The state of hyperglycemia causes nonenzymatic glycation of plasma proteins and glucose molecules suffer auto-oxidation causing an excessive production of ROS (Pereira & Martel, 2014). Increased levels of biomarkers of ROS damage and abnormalities in the antioxidant defenses exist in GDM patients and antioxidant intake appears to be able to attenuate GDM complications in the offspring (Pereira & Martel, 2014).

Intrauterine growth restriction

Intrauterine growth restriction is a pathologic condition where the placenta is not provided with sufficient maternal blood circulation which can lead to a non-healthy development of the foetus in the womb. Although this pathologic condition is considered as a multifactorial etiology pathology, several studies using oxidative stress biomarkers and cell membrane injury parameters, leaded to the conclusion that increased oxidative stress and intrauterine growth restriction are correlated (Pereira & Martel, 2014).

Spontaneous miscarriage and recurrent pregnancy loss

Spontaneous abortion can occur with an unintentional pregnancy termination before the foetus can be considered viable or when the fetus weight is less than 500g.
Some studies found relations between spontaneous abortion and an increase in MDA and lipid peroxides levels on placental tissue and related those with low activity of antioxidants such as catalase or glutathione peroxidase. An abnormal placentation causing damage in the STBs may be the cause of this high sensitivity to oxidative stress that contributes to spontaneous miscarriage (Pereira & Martel, 2014).

Preeclampsia

Preeclampsia, the most investigated complication of pregnancy, is a clinical syndrome of the systemic inflammatory response due to maternal endothelial cell dysfunction. It develops in the second or third trimester of pregnancy and is characterized by a generalized arteriolar constriction and intravascular depletion that can lead to poor perfusion of the maternal and fetal placenta. The shallow trophoblast invasion in preeclampsia leads to an increase of the neutrophil-endothelial adhesion and increases the production of TNFα, a cytokine produced mainly by macrophages. Although the causes of preeclampsia remains unknown, oxidative stress is considered as a feature of this disease since common biomarkers of oxidative stress such as protein carbonyls, MDA, lipids peroxides and DNA oxidation have been associated with preeclampsia (Pereira & Martel, 2014; Schoots et al., 2018).

3.2. Oxidative stress and xenobiotics

Pregnant mothers are exposed to a wide variety of foreign chemicals. This exposure is most commonly due to maternal medication, lifestyle factors such as smoking, drug

abuse, and alcohol consumption, or occupational and environmental sources. Foreign compounds may interfere with placental functions at many levels e.g. signaling, production and release of hormones and enzymes, transport of nutrients and waste products, implantation, cellular growth and maturation, and finally, at the terminal phase of placental life, i.e. delivery. Xenobiotics can also affect the placenta due to pharmaco/toxicodynamic responses to foreign chemicals, e.g. hypoxia (Myllinen et al., 2005).

Several foreign chemicals cause oxidative stress in placental tissue. Placentas exposed to tetrachlorodibenzo-p-dioxin (TCDD), cadmium and alcohol are in a state of oxidative stress (Myllinen et al. 2005). Many other foreign compounds, including halogenated insecticides, bisphenol A, arsenic, lead, polychlorinated biphenyl compounds (PCBs), metamphetamines and chlorpyrifos cause oxidative stress (Wells et al., 2016; Rock & Patisaul, 2018).

Objective

EVTs are essential for blastocyst implantation and establishment. They are also very important to the remodeling of the spiral arteries that will provide nutrients and oxygen from the mother to the foetus. Several pregnancy disorders and several xenobiotics are associated with increased oxidative stress levels, but not much is known concerning the effect of oxidative stress in EVTs, and more specifically in features of these cells associated with the placentation process.

Therefore, we aimed to understand the effects of increased oxidative stress levels on the placentation mechanism. For this, we used *tert*-butylhydroperoxide (TBH) as an oxidative stress inducer and HTR8/SVneo as a first trimester EVT cell line. We proceed to evaluate the effect of TBH on characteristics of EVTs involved in the placentation process (cell viability, proliferation rate, culture growth, nutrient (glucose) uptake, apoptosis rates and cell migration rates).

Materials and methods

1. Materials

Homogenization solution (KH_2PO_4 62.5 nM, $Na_2HPO_4.2H_2O$ 50 nM, Triton X-100 0.1%, pH=7), [^{3}H]-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE HealthcareGmbH, Freiburg, Germany), *tert*-butylhydroperoxide (TBH), decane, folic acid, glutathione reductase from baker's yeast (S. cerevisiae), RPMI 1640 medium, malondialdehyde, NADH (nicotinamide adenine dinucleotide reduced disodium salt hydrate), NADPH (nicotinamide adenine dinucleotide 2-phosphate reduced tetrasodium salt hydrate), hydrochloric acid, ethyl acetate, ethanol, paraformaldehyde, sodium hydroxide, sodium pyruvate, sulforhodamine B (SRB), 2-thiobarbituric acid (TBA), trichloroacetic acid sodium salt (TCA), 2,4-dinitrophenylhydrazine, guanidine hydrochloride, trypsin-EDTA solution, ethylenediaminetetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (Sigma, St. Louis, MO, USA); L-glutamine, L-asparagine, perchloric acid and Triton X-100 (Merck, Darmstadt, Germany); In Situ Cell Death Detection Kit, Fluorescein, 4,6 Diamino-2-phenylindole (DAPI) (ROCHE Diagnostics, Germany).

2. Methods

2.1. Cell culture

For our study we used HTR-8/SVneo cells that were kindly provided by Dr Charles H. Graham (Department of Anatomy & Cell Biology, Queen's University at Kingston, Canada) (Graham et al., 1993) and were used between passage numbers 124 and 152.

We maintained the cells in a humidified atmosphere of 5% CO_2 – 95% air, constant temperature of 37ºC and they were grown in RPMI 1640 medium with 5% (v/v) of fetal bovine serum (FBS) and 1% (v/v) of antibiotic/ antimycotic solution (AB-AM). We changed the culture medium every 2-3 days and the culture was split every 7 days. For periodic sub-culturing, the cells were removed enzymatically (0.05% trypsin-EDTA diluted in PBS, 3 min, 37ºC), split 1:4 ratio and sub-cultured in plastic culture dishes (21 cm^2, diameter 60 mm; Sarstedt, Nümbrecht, Germany).

For the LDH, SRB, ^{3}H-thymidine incorporation and wound-healing assays, the HTR8/SVneo were cultured in 24-well plates (2 cm^2, diameter 16 mm; TPP®, Trasadingen, Switzerland) with a 1:6 split ratio and were used after 4 days. For the TUNEL assay, we cultured the cells in coverslips inside a 24-well plate (2 cm^2, diameter 16 mm; TPP®) and the cells were cultured for 3 days. For the TBARS and protein carbonyl content assays, the

cells were cultured in 6-well plate (2 cm^2, diameter x mm; TPP®) and were used after 4 days in culture.

2.2. Induction of oxidative stress using TBH (tert-butyl hydroperoxide)

All the experiments were initiated when the cells reached 70-80% confluence. To mimic the effects of the oxidative stress, we exposed the cells to TBH (*tert*-butyl hydroperoxide) for 24h in culture medium without FBS. In the first series of experiments, we established the TBH concentration to use in the following studies, by evaluating oxidative stress levels using the TBARS and protein carbonyl groups assays. We were looking for a concentration of TBH that induced oxidative stress but did not cause cell death. For that, we first tested TBH 5 and 10 µM, but the cell death rate was too high. So, we then tested concentrations between 0.5 and 5 µM. After this, we decided that the best concentration for oxidative stress induction was 0.5 µM. Control cells were exposed for 24h to an identical concentration of decane in FBS-free culture medium.

2.3. Evaluation of oxidative stress

2.3.1. Quantification of thiobarbituric acid reactive substances (malondialdehyde) levels

The TBARS assay quantifies oxidative stress by measuring lipid peroxidation damage caused by free radicals in the cells. In our study, we used the TBARS (thiobarbituric acid reactive substances) assay to measure the lipid peroxidation, as next described.

HTR-8/SVneo cells were treated for 24h with TBH. At the end of the treatment, the homogenization solution was added and the cells were resuspended. From this resuspension, we transferred 300 µL to an Eppendorf and added 200 µL of TCA 50%. The samples were vortexed and rested for 5 min at room temperature to be next centrifugated at 10600 g for 2 min at 4ºC. Then, 300 µL of the supernatant were transferred to a new Eppendorf and 300 µL of TBA 1% (m/v) was added to it. The samples were then incubated for 40 min at 95ºC. After that, 300 µL of each sample was transferred to a 96-well culture plate and the absorbance at 535 nm was measured.

2.3.2. Quantification of protein carbonyl content

We measured the protein carbonyl content levels as a complement of the TBARS assay to confirm that TBH 0.5 µM affects another oxidative stress biomarker. The protein

carbonyl content is also used as a marker of protein oxidation. Protein carbonyls result from oxidation of amino acids or from binding of aldehydes produced from lipid peroxidation as a result of the oxidative stress. We used DNPH that reacts with the protein carbonyls producing hydrazones (DNP). We then quantify them spectrophotometrically at 370 nm.

HTR-8/SVneo cells were treated for 24h with TBH. At the end of the treatment, the extracellular medium was removed and the cells were washed with PBS solution. We next resuspended the cells with 400 µL of homogenization solution. 100 µL of the homogenized were added to an Eppendorf in duplicates and 200 µL of TCA 10% were added. The remain volume of the wells was used for Bradford method. We centrifuged the samples at 13.000 rpm for 2 min at 4ºC and the supernatant was discarded. Since we did duplicates, one was treated with 500 µL of DNPH and the other was treated with 500 µL HCL. Both rested for 1h being shaken every 10 min. After that, 500 µL of TCA 20% at 4°C were added, the samples were kept on ice for 15 min and then centrifuged at 13.000 rpm for 2 min at 4ºC. We then proceeded to wash the precipitate with ethanol:ethyl acetate (1:1) for three times, with centrifugations in between at 15300 g for 2 min at 4ºC, and we removed the supernatant by vacuum on the last washed. We next dissolved the sediment overnight with 500 µL of guanidine 6 M at 4ºC. The next day we centrifugated the samples at 960 g for 15 min to remove the non-dissolved material and we proceeded to transfer the cells to a 96 well plate. The absorbances were then measured with a plate reader at 340 nM.

2.4. Evaluation of the effect of oxidative stress on cellular characteristics

We evaluated the effect of oxidative stress on HTR8/SVneo cellular viability, proliferation rate, apoptosis rate, culture growth, migration rate and nutrient (glucose) uptake.

2.4.1. Cellular viability assessment by the LDH assay

To evaluate if oxidative stress has an effect on the viability of our cells, we quantified the lactate dehydrogenase (LDH) levels on the extracellular medium.

LDH is an oxidoreductase enzyme that is immediately released into the extracellular medium when the plasma membrane is damaged and its role in the cell is to catalyze the reversible oxidation of pyruvate to lactate using NADH. The equation of this reaction is:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+$$

The damage in the cell membrane is a sign of apoptosis, necrosis or other forms of cellular damage.

HTR-8/SVneo cells were treated for 24h with TBH (or decane). At the end of the treatment the extracellular medium was collected to an Eppendorf, and the extracellular LDH activity was quantified by measuring, in the culture medium, the oxidation of NADH at a wavelength of 340 nm during the reduction of pyruvate to lactate. Optical density values were determined for 2 min, and the rate of NADH oxidation was then calculated. To determine total LDH activity, cells from control cultures were solubilized with 0.5 ml 0.1% (v/v) Triton X-100 and placed for 30 min at 37 °C. The amount of LDH present in the extracellular medium was then calculated as a percentage of total LDH activity.

2.4.2. Cellular proliferation assessment by quantification of [^{3}H]-thymidine incorporation

To evaluate the effect of oxidative stress on the proliferation rate of the cells, we used the [^{3}H]-thymidine incorporation assay. [^{3}H]-thymidine is a radioactive DNA precursor that is incorporated on the new synthesized DNA chains during the S-phase of the cell cycle. The amount of [^{3}H]-thymidine detected by the scintillator correlates to the rate of the proliferation.

HTR-8/SVneo cells were treated for 24h with TBH (or decane) and were incubated with ^{3}H-thymidine during the last 5 h of the treatment. At the end of the treatment period, the medium was removed and the cells washed with 300 µL TCA 10% to remove the excess of ^{3}H-thymidine. The cells were then dissolved in 280 µL of NaOH 1M and rested for 15 min. The samples were then transferred to vials with 50 µL HCL 5 M and 2 mL scintillation liquid were added and the radioactivity was measured on a liquid scintillometer beta counter.

2.4.3. Culture growth assessment with the Sulforhodamine B (SRB) assay

To evaluate if oxidative stress has effect on the cell culture density, we used the sulforhodamine B (SRB) assay. This assay is based on the measurement of cellular protein content. SRB, an aminoxanthene dye for proteins, is added to the cell culture after the cells have been fixed with trichoroacetic acid (TCA). This aminoxanthene has two sulfonic groups that bind electrostatically to basic amino-acid residues under mild acidic and pH dependent conditions.

After being incubated for 24h with TBH (or decane), we added 62.5 µL TCA 50% to the HTR-8/SVneo cells and incubated the cells for 1h at 4ºC. The cells were then washed

with H_2O, 125 µL of SRB solution was added and the cells rested for 15 min. The cells were then washed with acetic acid and 375 µL of tris solution (pH=10,5) was added. We then transferred the samples to a 96-well plate in triplicate and the absorbances were measured with a plate reader at 540 nM.

2.4.4. Cellular migration quantification by the wound-healing assay

To evaluate if the oxidative stress would have any effect on the migration rate of the cells, we used the wound-healing assay. This assay consists on creating a wound on a monolayer cell culture and capturing images at regular time intervals during the cellular migration. We used these images as a means of comparison and to quantify the migration rates. We did a horizontal dash at the back of the plate and made the wound on the cell line with a micropipette tip before adding the treatment with TBH to the HTR-8/SVneo cells. We captured two photographs in each well, namely at 0h and 24h after the scratch. The images were then analyzed using the imageJ software.

2.4.5. Evaluation of apoptosis index with the TUNEL assay

To evaluate the effect of TBH upon the apoptosis rate we used the terminal deoxynucleotidyl transferasedUTP-mediated nick end labeling (TUNEL) assay. This assay allows the localization of apoptotic DNA fragmentation by means of the enzyme terminal deoxynucleotidyl transferase (TdT). This enzyme catalyzes the binding of deoxyribonucleoside triphosphates (dUTPs) to the free 3'- hydroxyl terminals of DNA ends that can be observed on a fluorescence microscopy. The amount of DNA fragments detected is an indicator of the apoptosis levels in the culture cells.

After being incubated for 24h with TBH (or decane), we removed the extracellular medium and added paraformaldehyde to fix the cells. After 30 min, we washed the cells with PBS to remove the excess and enhanced the cell permeability before adding the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland). In brief, 10 µL of the kit was added to the cells at a dark and humid atmosphere. 1h later, 20 µL of DAPI was added and the cells rested for 5 min. We then washed the cells with PBS + Triton X-100 solution two times with an interval of 5 min between washes. Immunofluorescence was visualized under a fluorescence microscope (Zeiss apopTome, Oberkochen, Germany). We prepared the microscope slides with glycerol and we captured 3 images from 3 different sites of each sample. The imageJ software was used to do the cell counting, and the apoptotic index was calculated as the percentage of apoptotic cells respective to total cell number.

2.4.6. Nutrient (glucose) uptake evaluation

To evaluate the effect of TBH upon glucose uptake by HTR8/SVneo cells, we measured the cellular uptake of ^{3}H-deoxy-D-glucose (^{3}H-DG). Briefly, after exposure to TBH (or decane) for 24h, the cells were washed with 300 µL GF-HBS buffer (composition in mM: 20 HEPES, 5 KCl, 140 NaCl, 2.5 MgCl2, 1 CaCl2, pH 7.4) at 37ºC. Then, we preincubated the cells in GF-HBS buffer for 20 min and then incubated them with ^{3}H-DG (a D-glucose analogue efficiently transported by GLUTs) 10 nM in 200 µL GF-HBS buffer for 6 min. We stopped the reaction by removing the incubation medium, rinsing the cells with 500 µL ice-cold GF-HBS buffer and added 300 µL Triton X-100 (0.1%) and Tris HCL 5 mM (pH=7,4) to let the samples overnight at 4ºC. We then transferred 250 µL of each sample to a vial containing 2 mL of scintillation liquid and intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

^{3}H-DG uptake was evaluated in the absence and presence of the facilitative glucose transporter 1 (GLUT1) inhibitor BAY-876. With the suppression of GLUT1 we wanted to understand the importance of GLUT1 in glucose uptake by HTR8/SVneo cells and to evaluate if TBH interferes with GLUT1-mediated and non-GLUT1-mediated transport.

2.5. Protein determination

The protein content was determined using the Bradford protein assay (Bradford, 1976), with human serum albumin as standard.

2.6. Statistical analysis

Our values are expressed as arithmetic means with standard error of mean (SEM). The value of n represents the number of replicates of at least two experiments performed.

The statistical analysis of the difference between two groups was made with *Student's t test* while the statistical analysis of the difference between more than two groups was made with the ANOVA test, followed by the *Student-Newman-Keuls* test. The differences are considered significative every time $p<0.05$.

Results

1. *Evaluation of the effect of TBH on oxidative stress levels*

In the first set of experiments, we tested the effect of distinct concentrations of TBH in order to choose the concentration to use in the following experiments. For this, we first evaluated the effects of TBH (0.5-10 μM) on lipid peroxidation levels (TBARS assay). As described in the Methods section, we were looking for a concentration that induces oxidative stress without causing cell death. So, we discarded all concentrations above 1 μM, as a significant cell loss was observed with these concentrations. As shown in Figure 3A, the amount of thiobarbituric acid reactive substances increased significantly after a 24h-treatment of the cells with TBH 0.5 μM and 1 μM. These concentrations of TBH were able to increase the lipid peroxidation levels, without causing a significant cell loss (evaluated by observation of the cell cultures at the microscope and by quantification of total cellular protein levels (results not shown)).

The protein carbonyl content assay was performed as a complement to the TBARS assay. Because the increase in TBARS levels was similar with TBH 0.5 and 1 μM, but the cell loss was higher with TBH 1 μM (as observed at the optical microscope), we decided to test the effect of TBH 0.5 μM on protein carbonyl levels. As shown in Figure 3B, the amount of protein carbonyls increased significantly after treatment of the cells with TBH of 0.5 μM. This is another indicator confirming that TBH 0.5 μM is able to increase oxidative stress levels in HTR8svneo cells and so, this treatment was used in the following experiments.

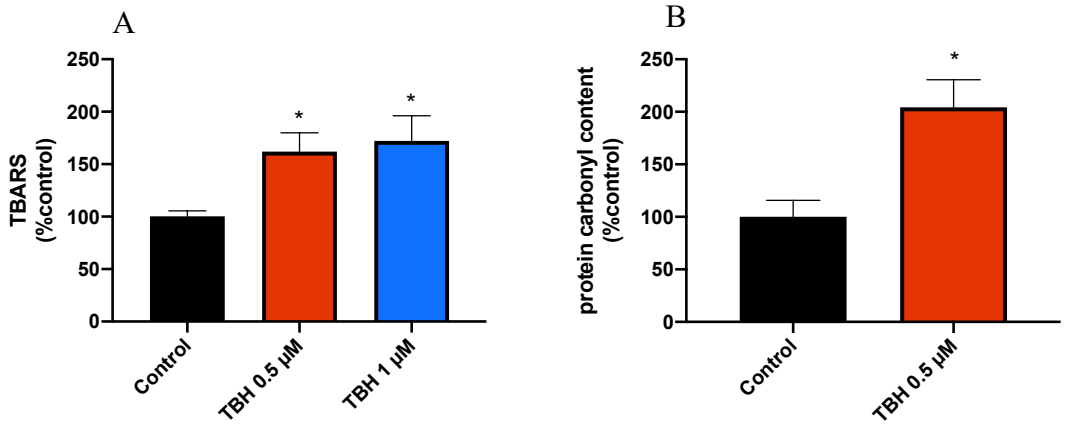

Figure 3. Quantification of TBARS levels (A) and protein carbonyl content (B) after exposure of HTR8/SVneo cell cultures to TBH*. The cells were exposed for 24h to TBH 0.5 μM (n=9) and 1 μM (n=18) Results are shown as arithmetic means±SEM. *significantly different from control (p<0.05)*

2. *Evaluation of the effect of TBH on cellular characteristics*

After establishing that we would use TBH 0.5 μM to induce oxidative stress in HTR8/SVneo cells, we evaluated its effect on cell viability, proliferation, glucose uptake, migration rates and apoptosis index.

2.1. Effect of TBH on cell viability

To evatuate the viability of HTR8/SVneo cells under oxidative stress conditions, we quatified the levels of the enzyme LDH on the extracelular medium. The cells were exposed to TBH (0.5 μM) for 24h as mentioned previously in our Methods. As shown in Figure 4, there was no significant change in LDH activity. So, TBH does not cause a significant change in the % of dead cells.

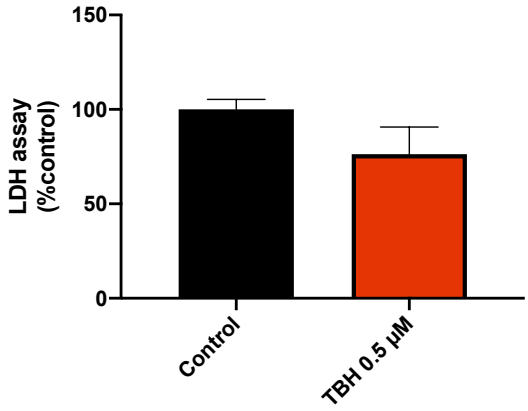

Figure 4. Effect of TBH on extracellular LDH activity. *HTR8/SVneo cells were exposed for 24h to TBH 0.5 μM (n=12) Results are shown as arithmetic means±SEM.*

2.2. Effect of TBH on cell proliferation

To evaluate the proliferation rate of HTR8/SVneo cells under oxidative stress conditions (TBH 0.5 µM for 24h), we used two methods: quantification of culture growth (SRB assay) and measurement of proliferation rates ([^{3}H]-thymidine incorporation).

The SRB assay measures the cellular total protein content while the [^{3}H]- thymidine incorporation assay allows us to understand the proliferation rate. As shown in Figure 5A and 5B, exposure of HTR8/SVneo cells to TBH (0.5 µM) significantly decreased the cellular protein content levels as well as the proliferation rates (by about 20% and 30%, respectively).

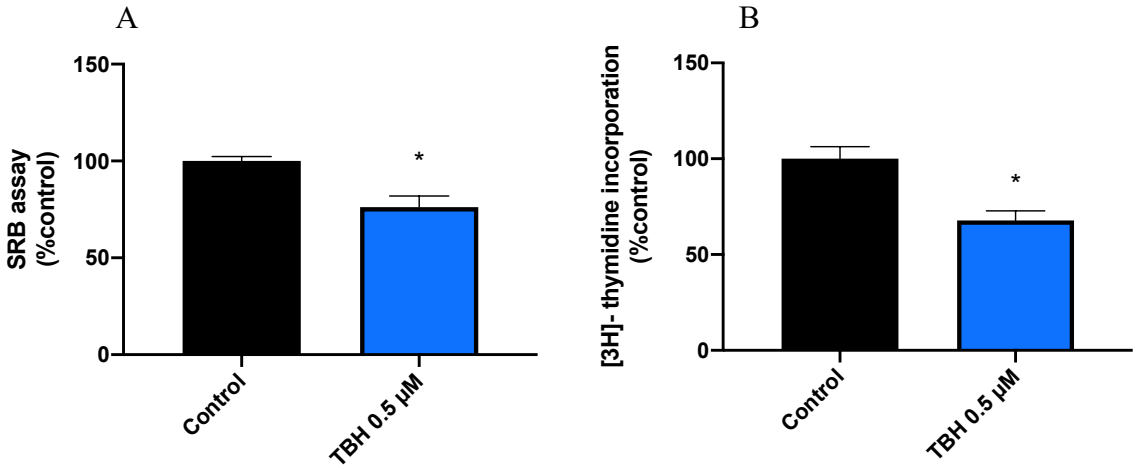

***Figure 5. Effect of TBH on cellular protein content (A) and [^{3}H]-thymidine incorporation levels (B)**. HTR8/SVneo cells were exposed for 24h to TBH 0.5 µM (n=6-8) Results are shown as arithmetic means±SEM. *significantly different from control (p<0.05)*

2.3. Effect of TBH on glucose cellular uptake

To evaluate the effect of oxidative stress on nutrient (glucose) uptake, we quantified ^{3}H-deoxy-D-glucose (^{3}H-DG; a non-metabolized glucose analogue efficiently transported across cell membranes by using GLUTs) cellular uptake and we also evaluated the effect of TBH on GLUT1.

As shown in Figure 6, ^{3}H-DG cellular uptake was not significantly changed after exposure to TBH (0.5 μM). We also tested the effect of the specific GLUT1 inhibitor Bay876, and the reduction in ^{3}H-DG uptake in its presence indicates that GLUT1 is involved in ^{3}H-DG uptake by these cells. On the other hand, when we combined the GLUT1 inhibitor (Bay876) with TBH, no significant change in ^{3}H-DG uptake, in comparison with Bay876 alone, was observed (Figure 4).

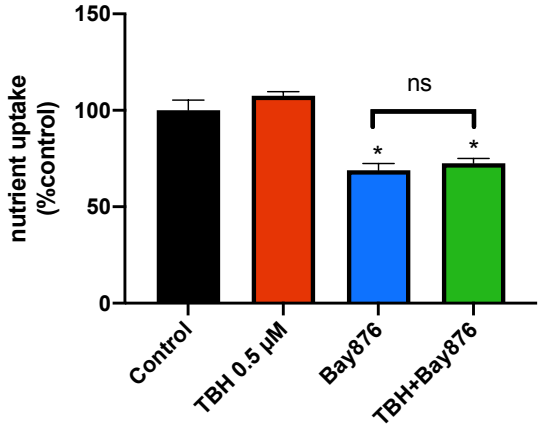

***Figure 6. Effect of TBH on ^{3}H-DG cellular uptake, in the presence and absence of GLUT1 inhibition**. HTR8/SVneo cells were exposed for 24h to TBH 0.5 μM (n=8). Results are shown as arithmetic means±SEM. *significantly different from control ($p<0.05$); ^{ns} not significantly different from each other*

2.4. Effect of TBH on cell migration rates

To evaluate the migration rates of HTR8/SVneo cells in an increased oxidative stress condition (TBH 0.5 µM for 24h), we used the wound-healing assay.

The images were captured in each well at 0h and 24h after the scratch and we quantified the area of the wound in order to verify if differences in cell migration exist. A smaller wound's area is equivalent to a higher migration rate. As shown in Figure 7, the migration rate decreased when the cells were exposed to TBH (0.5 µM).

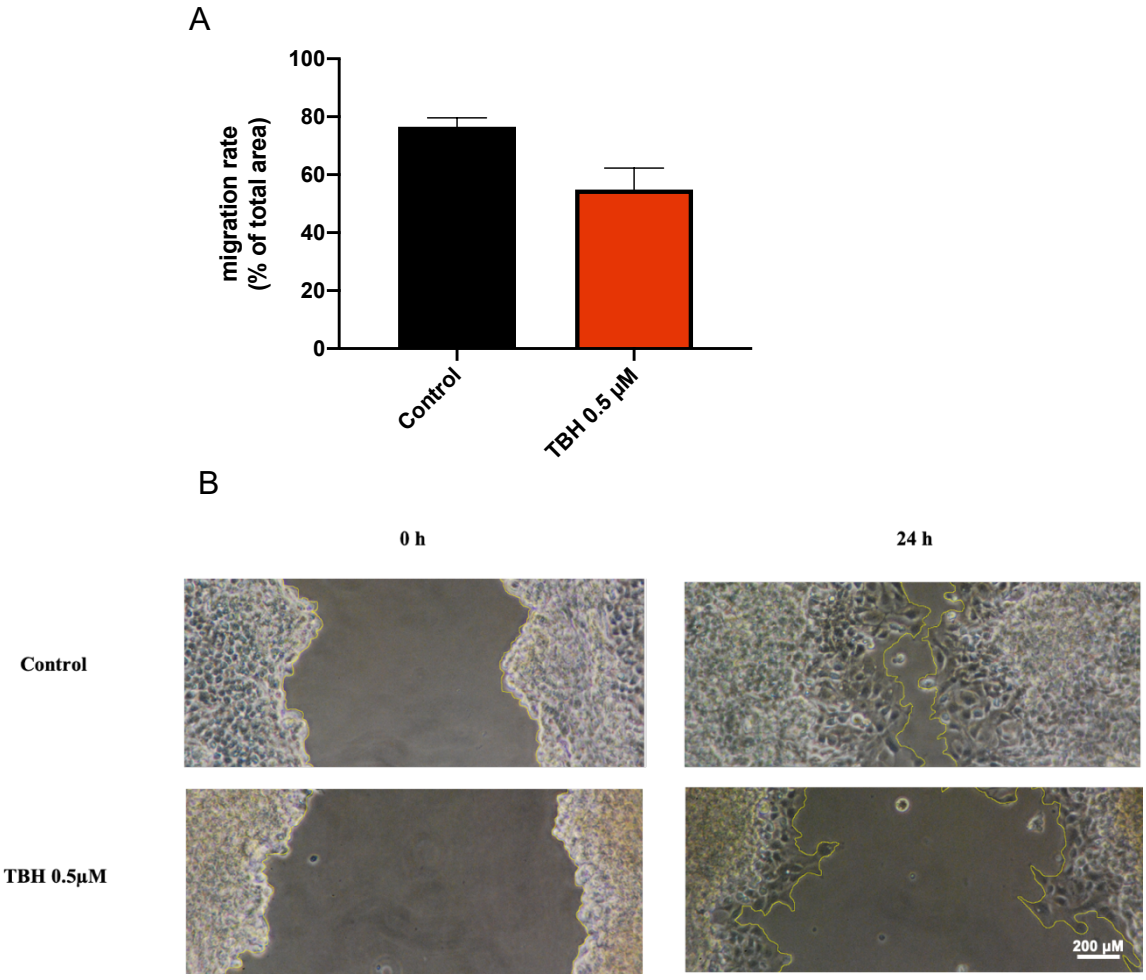

***Figure 7. Effect of TBH on cell migration rates**. HTR8/SVneo cells were exposed for 24h to TBH 0.5 µM (n=11-12). (A) Results are shown as arithmetic means±SEM.I; (B) Examples of images captured for the wound-healing assay.*

2.5. Effect of TBH on apoptosis rates

To evaluate the effect of TBH (0.5 µM) for 24h on apoptosis levels of HTR8/SVneo cells, we performed a TUNEL assay. As shown in Figure 8, the number of apoptotic cells increased when the cells were exposed to TBH. This means that under oxidative stress conditions, an increase in the % of HTR8/SVneo cells suffering programmed cell death is observed.

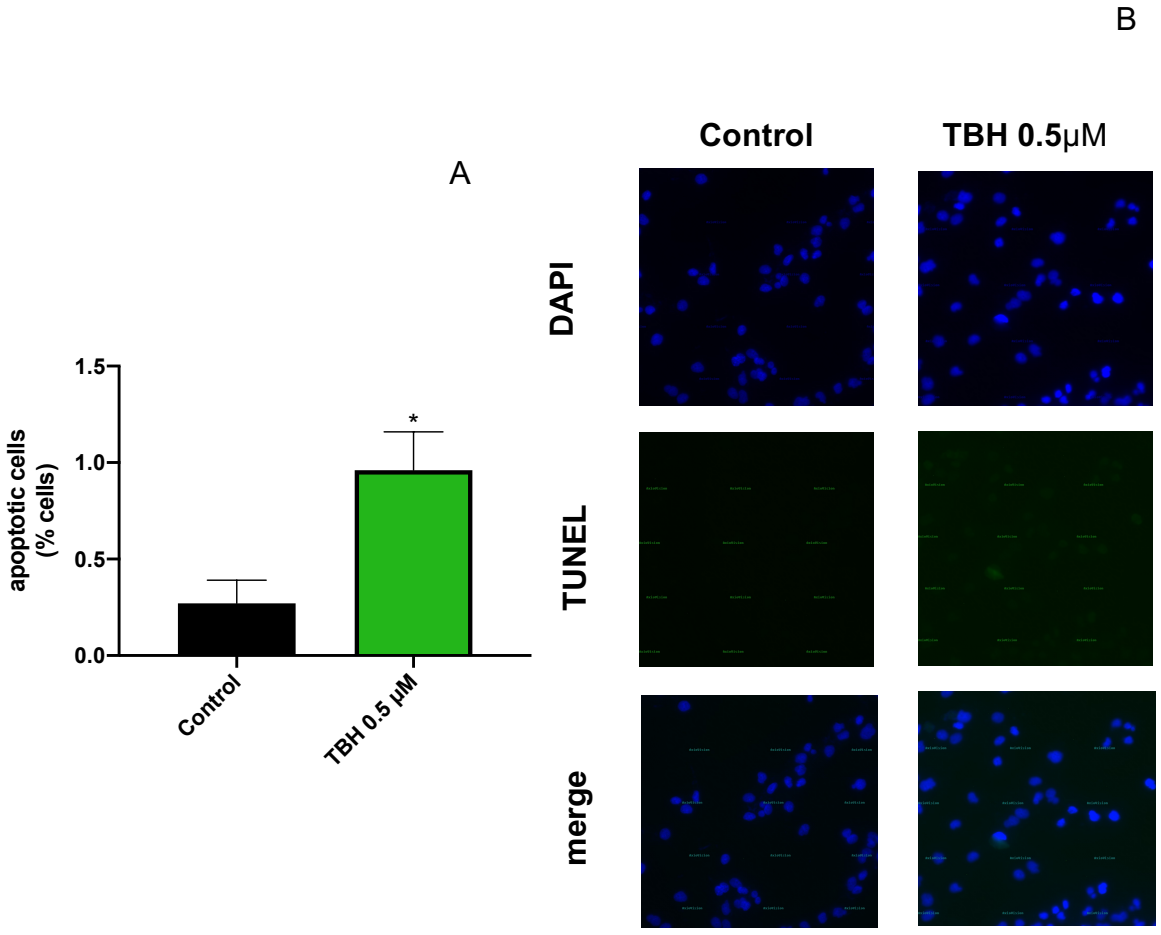

Figure 8. Effect of TBH on apoptosis rates. *HTR8/SVneo cells were exposed for 24h to TBH 0.5 µM. (A) Results are shown as arithmetic means±SEM (n=30). *significantly different from control (p<0.05); (B) HTR8/SVNneo cell's images captured for the TUNEL assay.*

Discussion and conclusions

During early pregnancy, EVTs must be precisely regulated so that several physiological processes correctly occur. These cells are responsible for the invasion of the maternal endometrium and for the construction and establishment of the vascular system between the mother and the foetus (Chang et al., 2018; Yang et al., 2019). Oxidative stress is defined by an imbalance between reactive oxygen species (ROS) and their respective antioxidants leading to an accumulation of ROS and consecutive cell and tissue damage (Jing et al., 2018). Pregnancy complications, including miscarriage, preeclampsia and gestational diabetes mellitus, are associated with abnormal EVT differentiation and invasion and are associated with high oxidative stress levels (Chang et al., 2018; Jing et al., 2018; Qi et al., 2020)

In our study, we investigated the effect of high oxidative stress conditions on the viability, proliferation, migration and apoptosis rate and on glucose uptake capacity of HTR8/SVneo cells. The HTR8/SVneo cell line is an established cell model of human EVT and our team has been using this cell line for some years (eg. Carletti et al., 2018; Correia-Branco et al., 2015; Correia-Branco et al., 2019). This cell line was developed from human first trimester EVT cells infected with SV40 Large T antigen and display an unlimited life-spam in culture while retaining the invasive and proliferative characteristics of these placental cells (Graham et al., 1993). As an oxidative stress inducer, we used TBH at a concentration of 0.5 μM. This concentration was shown to increase protein and lipid oxidative stress biomarkers (protein carbonyl and malondialdehyde content, respectively).

Our results suggest that treatment of the cells with TBH (0.5 μM) does not significantly change the % of necrotic cells, as evaluated with the measurement of extracellular LDH activity. This enzyme is leaked from the cell as a result of cell membrane damage, which occurs during necrosis. On the other hand, the apoptosis levels of HTR8/SVneo cells increased significantly in the presence of TBH. The accumulation of ROS in the cell leads to DNA and mitochondrial damage as well as endoplasmic reticulum stress which can activate the mechanisms that lead to cell death (Wang et al., 2020). The phenomenon of programed cell death guarantees that cells no longer functional can be eliminated without an inflammatory response and our results are compatible with previous data that show oxidative stress as a cause of cell apoptosis (Huppertz et al., 2006; Jing et al., 2018; Qi et al., 2020). Overall, these results suggest that the increase of oxidative stress caused by TBH (0.5 μM) stimulates apoptosis of the cells, without significantly causing non-programmed cell death (necrosis).

The proliferative characteristics of the EVTs are fundamental for this cell to play its role on placentation and foetal development. In our study, we analyzed the proliferation rate

in terms of DNA synthesis rates (by measuring [^{3}H]-thymidine incorporation) and in terms of culture growth with SRB levels. Our results suggest that the proliferation rate of EVT cells decreases significantly under oxidative stress conditions which means that one of the main functions of this cells can be compromised under increased oxidative stress levels.

Taking in consideration that fetal gluconeogenesis is minimal, the foetus is dependent on glucose delivery from the maternal circulation. Glucose supply is also essential for placenta development and transport of this sugar occurs by facilitative diffusion down its concentration gradient mediated by glucose transporters (GLUTs) (Correia-Branco et al., 2015; Staud & Karahoda, 2018). We used ^{3}H-DG in our glucose transport studies and by using the specific GLUT1 inhibitor (Bay876) we could verify that GLUT1 plays an important role in the uptake of ^{3}H-DG by this cell line. Since GLUT1 is highly expressed in this cell line (Correia-Branco et al., 2015), our results suggest that this might be the main GLUT used by EVT cells. We also observed that ^{3}H-DG uptake was not affected by exposure of the cells to TBH (0.5 μM), both in the absence and presence of Bay876. So, we can conclude that the increase in oxidative stress levels affected neither GLUT1-mediated nor non GLUT1-mediated ^{3}H-DG uptake by the HTR8/SVneo cell line.

During the implantation of the placenta, EVTs are very invasive and their migration rate is high in order to penetrate the endometrium and vessel walls of the mother (Moser et al., 2018; Zheng et al., 2020). Our results concerning the migration rates of HTR8/SVneo show that increased oxidative stress levels negatively affect their migration ability, suggesting that oxidative stress, by interfering with the migration ability of EVTs, can compromise the placentation and the oxygen and nutrient supply.

In conclusion, our results show that TBH (0.5 μM, 24h) is able to increase oxidative stress levels in HTR8/SVneo cells, as evaluated by the increase in lipid peroxidation and protein carbonylation, which are two distinct oxidative stress biomarkers. These conditions of increased oxidative stress levels were found to decrease the proliferation rates of HTR8/SVneo cells, their migratory capacity and to increase apoptosis index. So, our results show that oxidative stress interferes with EVT characteristics essential for the placentation process, and so this direct effect of oxidative stress on EVT cells may contribute to the association between oxidative stress and pregnancy disorders.

Future work perspectives

Several studies have previously shown that antioxidant molecules were able to reduce oxidative stress levels in HTR8/SVneo cells (Barnea et al., 2016; A. D. Bolnick et al., 2017; J. M. Bolnick et al., 2015; Zou et al., 2014). However, little is known concerning the ability of antioxidants to reverse the effects of high oxidative stress levels in these cells. Since antioxidant therapy is commonly used during pregnancy to prevent pregnancy disorders, several antioxidants such as vitamin C, vitamin E and dietary polyphenols could be tested to evaluate if they are able to reverse the consequences of high oxidative stress levels in HTR8/SVneo cells.

References

Aouache, R., Biquard, L., Vaiman, D., & Miralles, F. (2018). Oxidative stress in preeclampsia and placental diseases. *International Journal of Molecular Sciences*, *19*(5).

Barnea, E. R., Vialard, F., Moindjie, H., Ornaghi, S., Dieudonne, M. N., & Paidas, M. J. (2016). PreImplantation Factor (PIF*) endogenously prevents preeclampsia: Promotes trophoblast invasion and reduces oxidative stress. *Journal of Reproductive Immunology*, *114*, 58–64.

Bolnick, A. D., Bolnick, J. M., Kohan-Ghadr, H.-R., Kilburn, B. A., Pasalodos, O. J., Singhal, P. K., … Drewlo, S. (2017). Enhancement of trophoblast differentiation and survival by low molecular weight heparin requires heparin-binding EGF-like growth factor. *Human Reproduction (Oxford, England)*, *32*(6), 1218–1229.

Bolnick, J. M., Kilburn, B. A., Bolnick, A. D., Diamond, M. P., Singh, M., Hertz, M., … Armant, D. R. (2015). Sildenafil stimulates human trophoblast invasion through nitric oxide and guanosine 3',5'-cyclic monophosphate signaling. *Fertility and Sterility*, *103*(6), 1587-1595.e2.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254.

Carletti, J. V., Correia-Branco, A., Silva, C. R., Andrade, N., Silva, L. O. P., & Martel, F. (2018). The effect of oxidative stress induced by tert-butylhydroperoxide under distinct folic acid conditions: An in vitro study using cultured human trophoblast-derived cells. *Reproductive Toxicology*, *77*, 33–42.

Chang, C. W., Wakeland, A. K., & Parast, M. M. (2018). Trophoblast lineage specification, differentiation and their regulation by oxygen tension. *Journal of Endocrinology*, *236*(1), R43–R56.

Correia-Branco, A., Azevedo, C. F., Araújo, J. R., Guimarães, J. T., Faria, A., Keating, E., & Fátima Martel. (2015). Xanthohumol impairs glucose uptake by a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) and impacts the process of placentation. *Molecular Human Reproduction*, *21*(10), 803–815.

Correia-Branco, A., Keating, E., & Martel, F. (2019). Placentation-related processes in a human first-trimester extravillous trophoblast cell line (HTR-8/SVneo cells) are affected by several xenobiotics. *Drug and Chemical Toxicology*, *42*(5), 541–545.

Forbes, K., & Westwood, M. (2010). Maternal growth factor regulation of human placental development and fetal growth. *Journal of Endocrinology*, *207*(1), 1–16.

Graham, C. H., Hawley, T. S., Hawley, R. G., Macdougall, J. R., Kerbel, R. S., Khoo, N., &

Lala, P. K. (1993). Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Experimental Cell Research*.

Gude, N. M., Roberts, C. T., Kalionis, B., & King, R. G. (2004). Growth and function of the normal human placenta. *Thrombosis Research*, *114*(5-6 SPEC. ISS.), 397–407.

Gupta, R. K., & Gupta, R. C. (2017). Placental toxicity. *Reproductive and Developmental Toxicology*, 1301–1325.

Huppertz, B., Kadyrov, M., & Kingdom, J. C. P. (2006). Apoptosis and its role in the trophoblast. *American Journal of Obstetrics and Gynecology*, *195*(1), 29–39.

James, J. L., Carter, A. M., & Chamley, L. W. (2012). Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta*, *33*(5), 327–334.

Jing, L., Ding, Z., Yang, Y., Baohong, M., Wang, Y., & Xiaoying, X. (2018). Lycium barbarum polysaccharides protect human trophoblast HTR8/SVneo cells from hydrogen peroxide-induced oxidative stress and apoptosis. *Molecular Medicine Reports*, *18*(3), 2581–2588.

Myllynen, P., Pasanen, M., Pelkonen, O. (2005) Human placenta: a human organ for developmental toxicology research and biomonitoring. *Placenta,* 26(5), 361-71.

Moser, G., & Huppertz, B. (2017). Implantation and extravillous trophoblast invasion: From rare archival specimens to modern biobanking. *Placenta*, *56*, 19–26.

Moser, G., Windsperger, K., Pollheimer, J., de Sousa Lopes, S. C., & Huppertz, B. (2018). Human trophoblast invasion: new and unexpected routes and functions. *Histochemistry and Cell Biology*, *150*(4), 361–370.

Pereira, A. C., & Martel, F. (2014). Oxidative stress in pregnancy and fertility pathologies. *Cell Biology and Toxicology*, *30*(5), 301–312.

Qi, L., Jiang, J., Zhang, J., Zhang, L., & Wang, T. (2020). Curcumin protects human trophoblast HTR8/SVneo cells from H2O2-induced oxidative stress by activating nrf2 signaling pathway. *Antioxidants*, *9*(2).

Rock, K.D., Patisaul, H.B. (2018). Environmental Mechanisms of Neurodevelopmental Toxicity. *Curr Environ Health Rep,* 5(1):145-157.

Schoots, M. H., Gordijn, S. J., Scherjon, S. A., van Goor, H., & Hillebrands, J. L. (2018). Oxidative stress in placental pathology. *Placenta*, *69*, 153–161.

Staud, F., & Karahoda, R. (2018). Trophoblast: The central unit of fetal growth, protection and programming. *International Journal of Biochemistry and Cell Biology*, *105*, 35–40.

Syme, M. R., Paxton, J. W., & Keelan, J. a. (2004). Human Placenta. *Clinical Pharmacokinetics*, *43*(8), 487–514.

Turco, M. Y., & Moffett, A. (2019). Development of the human placenta. *Development*

(Cambridge), *146*(22), 1–14.

Wang, P., Huang, C. xi, Gao, J. jun, Shi, Y., Li, H., Yan, H., … Zhang, Z. (2020). Resveratrol induces SIRT1-Dependent autophagy to prevent H2O2-Induced oxidative stress and apoptosis in HTR8/SVneo cells. *Placenta*, *91*(December 2019), 11–18.

Wells, P.G., Bhatia, S., Drake, D.M., Miller-Pinsler, L. (2016). Fetal oxidative stress mechanisms of neurodevelopmental deficits and exacerbation by ethanol and methamphetamine. *Birth Defects Res C Embryo Today,* 108(2):108–30.

Windsperger, K., Dekan, S., Pils, S., Golletz, C., Kunihs, V., Fiala, C., … Pollheimer, J. (2017). Extravillous trophoblast invasion of venous as well as lymphatic vessels is altered in idiopathic, recurrent, spontaneous abortions. *Human Reproduction*, *32*(6), 1208–1217.

Wong, F., & Cox, B. J. (2017). Cellular analysis of trophoblast and placenta. *Placenta*, *59*, S2–S7.

Wu, F., Tian, F. J., & Lin, Y. (2015). Oxidative stress in placenta: Health and diseases. *BioMed Research International*, *2015*.

Yang, C., Lim, W., You, S., & Song, G. (2019). 4-Methylbenzylidene-camphor inhibits proliferation and induces reactive oxygen species-mediated apoptosis of human trophoblast cells. *Reproductive Toxicology*, *84*(December 2018), 49–58.

Zheng, L., Huang, J., Su, Y., Wang, F., Kong, H., & Xin, H. (2020). Overexpression of tissue factor pathway inhibitor 2 attenuates trophoblast proliferation and invasion in preeclampsia. *Human Cell*, *33*(3), 512–520.

Zou, Y., Zuo, Q., Huang, S., Yu, X., Jiang, Z., Zou, S., … Sun, L. (2014). Resveratrol inhibits trophoblast apoptosis through oxidative stress in preeclampsia-model rats. *Molecules (Basel, Switzerland)*, *19*(12), 20570–20579.