Chapter

Autophagy in Preeclampsia

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Abstract

Autophagy may be involved in gestation complicated by preeclampsia (PE) due to the presence of placental lesions caused by hypoxia at fetomaternal interphase. Autophagy is a lysosomal degradation pathway, removing protein aggregates and organelles damaged and thereby maintaining cell integrity. In preeclampsia, deficient myometrial penetration by extravillous cytotrophoblast occurs during the first trimester of pregnancy, leading to placental insufficiency. Several placental functions, like nutrient and oxygen input to the fetus during pregnancy, might benefit or even rely on autophagy and related material recycling within the cell. Deficiency in autophagy mechanism has been correlated to inflammatory responses. Autophagy is regulated during placentation and appears to be a possible factor in the development of preeclampsia. In this chapter, we intend to discuss evidence on autophagy pathway in pregnancy and the crosstalk between autophagy and inflammation in preeclampsia.

Keywords: preeclampsia, autophagy, inflammasome, placenta, inflammation

1. Introduction

Autophagy is an intracellular degradation system conserved among eukaryotes [1] characterized as a natural defense mechanism capable to reduce damages related to inflammatory responses and infectious, neoplasia and degenerative diseases. Defects in this process are directly linked to several diseases [2, 3]. The main role of autophagy is to maintain cellular homeostasis by recycling intracellular materials. Cells under starvation or with organelle damage trigger the autophagy mechanism in order to clean their cytoplasm and restore a source of energy [4–6].There are three types of autophagy: microautopahgy, chaperone-mediated autophagy, and macroautophagy [7]. The most characteristic process of autophagy is the macroautophagy. This process is characterized by the creation of a double membrane compartment in the cytoplasm called phagophore. This structure develops into an autophagosome, a double lipid bilayer membrane-bound structure. The autophagosome merges with lysosomes leading to the autolysosome formation. Continuing the process of autophagy, the autolysosome is finally degenerated, releasing in the cytosol a number of monomers that can be reused by the cell as important sources of energy (**Figure 1**) [8, 9].

The beginning of the autophagy process is dependent on the inhibition of the mammalian target of rapamycin (mTOR) and then autophagy proteins become crucial to the completion of the cascade generated [5, 10]. In this manner, cells under sufficient nutrition or without structural damages maintain a state of activation of mTOR, keeping blocked the mechanism of autophagy. After being triggered, mTOR provides a catalytic subunit by two distinct multiprotein complexes,

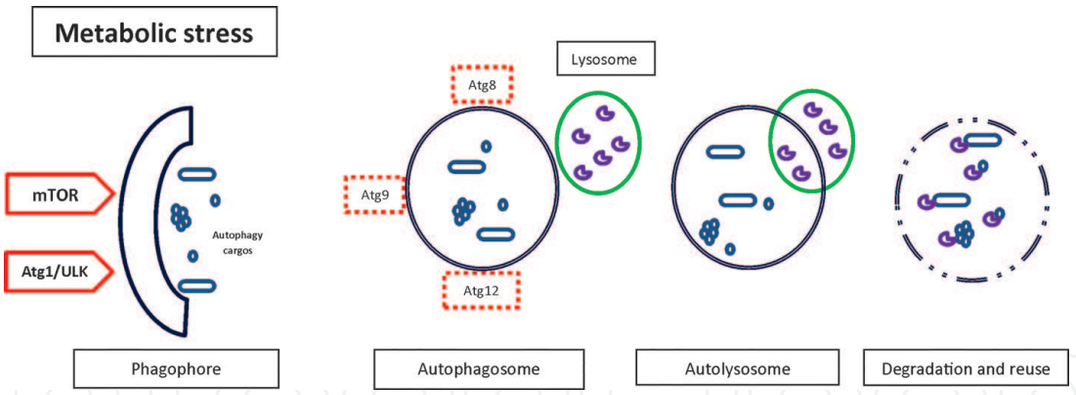

Figure 1.
Autolysosome formation. The process starts with a development of a double membrane named phagophore. This structure develops into an autophagosome, merging with lysosomes to form the autolysosome. During the process of autophagy, the autolysosome is degraded, releasing monomers into the cytosol, which can be reused by the cell as a source of energy. Source: own authorship.

mTORC1 (mechanistic target of rapamycin complex 1), and mTORC2 (mechanistic target of rapamycin complex 2). Some proteins are common for both complexes (GβL and Deptor), but others reflect specific functions for each of them, as raptor for mTORC1 and rictor for mTORC2 [11]. Regarding functions, mTORC1 coordinates the synthesis of lipids and proteins to promote cell growth, while mTORC2 acts in the cytoskeletal actin control [12]. Therefore, mTORC1 complex finally establishes the autophagy process via ULK1 (mammalian ortholog of yeast autophagy-related gene 1) kinase activity. The process of autophagosome formation involves about 38 Atgs (autophagy-related genes), and the initial process of autophagy in mammals is controlled by Atg1/ULK complex, which consists in ULK1/2 (UNC-51-like kinase 1), mAtg13, FIP200 (interaction protein of 200KD, homolog of the yeast Atg17), and Atg101. During nutrient rich conditions or in the presence of cell integrity, mTORC1 phosphorylates and inhibits ULK1/2 and mAtg13, deregulating the contact between ULK and AMPK (AMP-activated protein kinase), a kinase with activation effect on ULK1. But under altered conditions, mTORC1 is inhibited, and the activation of ULK1/2 leads to phosphorylation and activation of mAtg13 and FIP200 [13].

Atg plays crucial roles as ubiquitin-like protein conjugation system that mediates protein lipidation and participation in autophagy-specific protein kinase complexes [14, 15] for accomplishment of autophagy. Atg1 and Atg12-Atg5 complexes are essential for the autophagic machinery. Another important gene is Atg8 (a yeast autophagy-related gene ortholog of LC3), a membrane marker during the formation of autophagosome. Atg9 transmembrane protein and regulators of its trafficking (Atg2 and Atg18) participate in the expansion of phagophore after the formation of the Atg1 complex. There are two systems, Atg12 (Atg5, Atg7, Atg10, Atg12, and Atg16) and Atg8 (Atg3, Atg4, Atg7, and Atg8), which are responsible for expansion of the vesicle [1, 16].

The establishment of the autophagy is also dependent of two proteins: beclin-1 protein and LC3 (light chain protein 3) [17, 18]. Beclin-1 (homolog of yeast Apg6/Vps30) promotes the recruitment of membranes to form the autophagosome, nucleation of the autophagic vesicle, and recruitment of proteins from the cytosol [19]. Three isoforms of LC3 have been described: LC3A, LC3B, and LC3C. After synthesis and activation (LC3-I to LC3-II), LC3-II for incorporating cytosolic denatured proteins and damaged organelles into the autophagosome [20, 21]. The relative amounts of LC3-II reflect autophagic activity [22]. Considering the autophagy pathway, LC3-II has been proposed to be used to measure autophagy activity.

Defective autophagy caused by mutations or genetic alterations can lead to various clinical syndromes [23], such as static encephalopathy of childhood with neurodegeneration in adulthood (SENDA), Vici syndrome, hereditary spastic paraparesis, Parkinson's disease, lysosomal storage disorders, cancer, and Crohn's disease.

As a lysosomal degradation pathway, removing protein aggregates and organelles damaged, maintaining cell integrity, autophagy may be impaired in pregnant women with preeclampsia (PE) due to the presence of placental lesions caused by hypoxia/ischemia [24]. In preeclampsia, there is deficient myometrial penetration by extravillous cytotrophoblast during the first trimester of pregnancy, leading to placental insufficiency [25]. On the other hand, it has been reported that proteins related to autophagy, LC3-II, and beclin-1 are present in trophoblastic villi during normal pregnancy, and high levels of LC3-II are present in the placenta of pregnant women with severe preeclampsia [26]. The induction of hypoxia on choriocarcinoma cell line JEG-3 with the purpose of exploring the mechanism of regulatory proteins involved in autophagy showed a slight increase in the expression of LC3-II, with a reduction in beclin-1. Treatment of these cells with TNF-α induced a significant increase in the expression of LC3-II without modifying the expression of beclin-1. The results suggest that the increased autophagic activity mediated by LC3-II may be involved in the pathophysiology of preeclampsia [26].

Studies with autophagy in the placenta are scarce, and most of the work done employs cell lines or cultures of trophoblast cells in vitro to evaluate autophagy induced by nutrient deprivation and oxygen [27].

The systemic inflammatory response exacerbated in preeclampsia seems to be related to the release of substances capable of inducing inflammation as membrane fragments of syncytiotrophoblast, fetal DNA, soluble microparticles derived from leukocytes, and inflammatory cytokines in plasma of pregnant women, causing activation of cells of innate immunity [28–30]. Other cellular components present in plasma, such as protein derivatives, polysaccharides, and lipids, as well as extracellular matrix products are named "damage-associated molecular patterns" (DAMPS) and are considered important modulators of the inflammatory response. DAMPs are represented by molecules like uric acid, reactive oxygen intermediates, heat shock proteins (Hsp) [31], proteins released from dead cells, as the high mobility group box 1—HMGB1 [32], and products released from the extracellular matrix, such as fibronectin and hyaluronan [33, 34]. Both protein hsp70 [35, 36] and hyaluronan [37, 38] are elevated in plasma of pregnant women with preeclampsia and may be associated with systemic inflammation and oxidative stress. However, the role of these factors in the pathophysiology of preeclampsia is not well understood. A major cause of preeclampsia is the accumulation of reactive oxygen species (ROS), resulting in impaired antioxidant protection and activation of autophagy. According to research in trophoblasts, expression of LC3 and beclin-1 and the formation of autophagosomes are higher than in normal placentas, suggesting that autophagy is regulated during placentation and appears to be a possible factor in the development of preeclampsia [39].

2. Autophagy and placenta

The placental development requires multiples roles of autophagy. Experimental studies suggest that autophagy plays important functions in survival of neonates during nutritional deficiency at the early stage of birth [40]. Moreover, it was seen that the growth and remodeling of cervical fascia progress by autophagy regulation. Meanwhile, it has not been reported that autophagy affects differentiation

of trophoblasts in pregnant women by LC3-II and beclin-1, markers which are analyzed and compared between term placentas, and in their first trimester of gestation [39]. Autophagy is essential for placental development and for maintaining pregnancy, and the disruption of autophagy in extravillous trophoblast (EVT) contributes to hypoplastic placentation. Placentas of patients with preeclampsia present high levels of autophagy, with lower LC3 activation, and higher apoptosis than normal pregnancies. When induced by external factors such as hypoxia, autophagy directly affects trophoblast infiltration during normal placental development [41].

The study of autophagy markers in preeclampsia demonstrated that there are different patterns during normal pregnancy and preeclampsia, in part, because of the environmental factors, like hypoxia. Cells exposed to hypoxic conditions demonstrated higher levels of LC3, beclin-1, and the autophagosome formation when compared to normal placentas. Saito and Nakashima [24] reported that poor placentation is induced by decreased infiltration of trophoblasts due to abnormal processing for autophagy, which is activated by soluble endoglin (sENG). Inactivation of autophagy represses trophoblast infiltration and vascular remodeling due to excessive hypoxia, causing poor placentation, as observed in preeclampsia [26]. Hung and collaborators showed that autophagy decreased with advancing of gestational age in placentas of normotensive women, through analyses of changes in LC3-II and p62 according to gestational weeks [42].

The determination of p62 levels also has a few reports and in addition with the analysis of LC3-II [2, 14, 43] may be important to determine autophagic status in the villous tissue.

This important finding has been the basis of several investigations on oxidative stress, an alteration identified in placental related disorders, and whether this oxidative stress is able to induce autophagy activation on placental explants. We developed an in vitro study with the objective of evaluating the effect of oxidative stress induced by hydrogen peroxide (H_2O_2) on the occurrence of autophagy activation in placental explants of pregnant women at term (39–40 weeks), without clinical or obstetric disorders identified and undergone to elective cesarean section. In the methodology, we intended to reproduce experimentally the pathophysiology of obstetric complications related to placental dysfunction caused by uterine circulatory alterations. These alterations are responsible for the establishment of the hypoxia-reoxygenation phenomenon also called as ischemia-reperfusion injury and consequent production of (ROS).

The results showed the higher gene expression of LC3-II, beclin-1, and p62 detected in cultures exposed to different concentrations of H_2O_2 and demonstrated that the oxidative stress generated was able to induce autophagy in placental explants (**Figure 2**). Material and methods of this experiment are shown as supplementary material to this chapter.

Gene expression of LC3-II (**Figure 2A**) was increased in tissues exposed to the concentration of 1000 μM of H_2O_2 compared to the non-exposed cultures, which means that autophagy was more activated in this concentration, in response to the oxidative effect. Gene expression of beclin-1 and p62 (**Figure 2B** and **C**) increased in front of increasing concentrations of H_2O_2 with the maximum value in 1000 μM of H_2O_2, showing statistical difference ($p < 0.05$) when compared to controls.

In the present study, the higher gene expression of LC3-II, beclin-1, and p62 detected in cultures exposed to H_2O_2 demonstrated that the oxidative stress generated was able to induce autophagy in placental explants.

The high expressions of mRNA for LC3-II and TNF-α (**Figure 3A** and **B**) demonstrated in our study corroborate with other reports, showing that placental

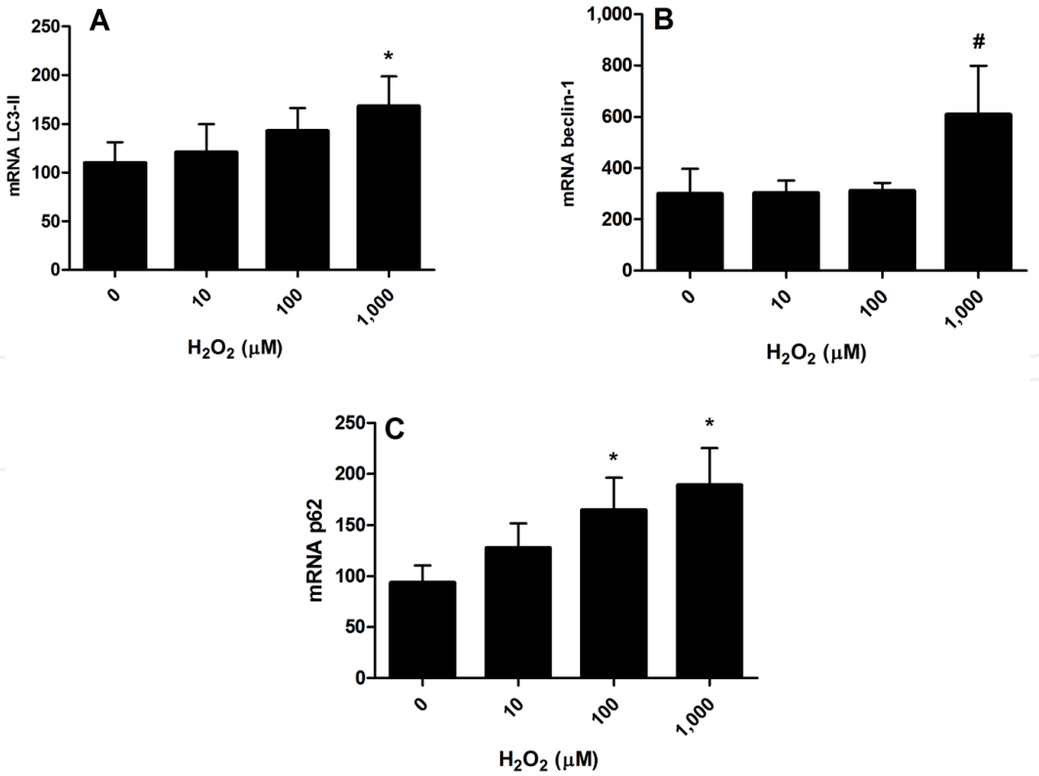

Figure 2.
*Activation of autophagy in cultures exposed to different concentrations of hydrogen peroxide. mRNA expression of LC3-II (A), beclin-1 (B), and p62 (C) in placental explants. Results expressed as mean ± SD. * ($p < 0.05$) vs. 0; # ($p < 0.05$) vs. 0, 10, 100 μM (ANOVA). Source: own authorship.*

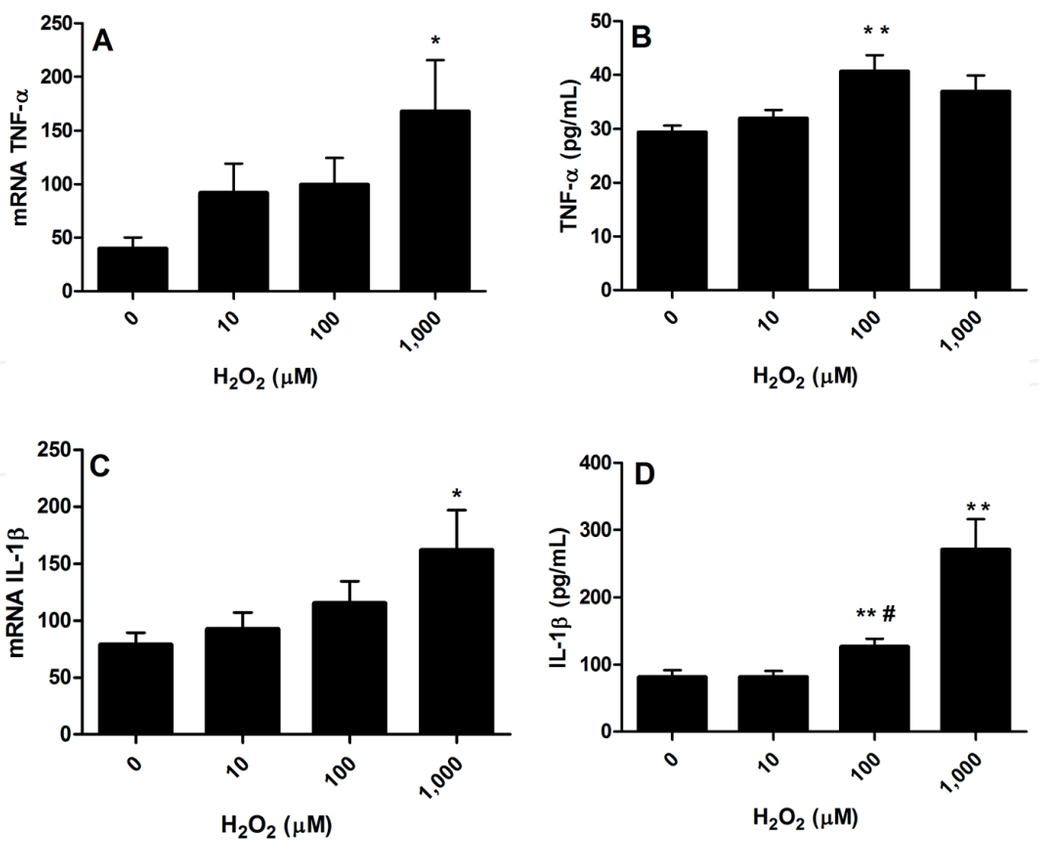

Figure 3.
*Pro-inflammatory cytokine profile in cultures exposed to different concentrations of hydrogen peroxide. Gene and protein expression of pro-inflammatory cytokines TNF-α(A/B) and IL-1β (C/D) in placental explants. Results expressed as mean ± SD. * ($p < 0.05$) vs. 0 (ANOVA). Source: own authorship.*

hypoxic environment and the presence of TNF-α caused a positive regulation in the autophagy-related protein LC3-II in a trophoblastic cell line [26].

Gene expressions of TNF-α and IL-1β were higher in explants cultured with 1000 μM of H_2O_2 with significant difference ($p < 0.05$) to culture without H_2O_2 (**Figure 3A** and **C**). The protein expressions of these cytokines (**Figure 3B** and **D**) were higher in the supernatants from concentrations of 100 to 1000 μM of H_2O_2, with difference ($p < 0.05$) to non-exposed and cultures with 10 μM of H_2O_2. The protein expression of IL-1 β was higher in 1000 μM of H_2O_2 ($p < 0.05$) than in the culture of 100 μM of H_2O_2 (**Figure 3D**).

The similar patterns of p62, beclin-1, and LC3-II gene expressions suggest the production of mRNA for the p62 protein transcription, which will be degraded during autophagy activation. This protein plays a significant role on the regulation of oxidative stress, degenerative diseases, and carcinogenesis [44].

3. Autophagy and inflammasomes

Inflammasome is a molecular platform composed of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins. It is required for the activation of caspase-1 and subsequent maturation of the pro-inflammatory cytokines interleukin-1β and IL-18 [45]. NLRs respond to different endogenous or exogenous stimuli and activate caspase-1. During inflammation, these pro-inflammatory molecules are maturated in inflammasomes, which are in part regulated by the autophagy mechanism.

Actually, autophagy can control endogenous inflammasome activators, such as pro-IL-1β, which regulates the secretion of IL-1β and IL-18, thereby preventing an exaggerated inflammation [9, 45, 46].The activation of the inflammasome NLRP3 occurs via two signals: the first signal is provided by NF-kB activators and is a prerequisite for inflammasome activation via NLRP3 expression in macrophages [47]; the second signal activates NLRP3 inflammasome directly and involves host-derived adenosine triphosphate (ATP), uric acid crystals, bacterial toxins, or particulate matter (**Figure 4**) [48].

Some studies have demonstrated a mutual relationship between autophagy and inflammasomes. Autophagy negatively regulates inflammasome activation. Autophagy induction is dependent on the presence of specific inflammasome sensors, inflammasomes are ultimately degraded by autophagosomes via the selective autophagic receptor p62, and autophagy plays a role in the biogenesis and secretion of the pro-inflammatory cytokine IL-1β [45, 9, 49].

The role of autophagy in inflammasome regulation may depend on the context of danger signal. In the absence of a danger signal, autophagy can act removing IL-1β and inflammasome components while maintaining cellular homeostasis. In the presence of a danger signal, autophagy may act initially as a secretory pattern to diffuse inflammation while preventing cell death and pyroptosis. Recent studies showed that macrophages may activate autophagy in response to inflammasome activation, as a way to delay the onset of pyroptosis. According to the authors, the inhibition of autophagy resulted in increased activation of pyroptosis and the impact of these types of cell death regulation by autophagy need to be more studied on inflammatory process [50]. When genes of autophagy regulator Atg 16L1 or Atg7 are deleted or a chemical inhibitor of autophagy is applied, LPS-dependent inflammasome activation occurs suggesting that autophagy controls inflammasome activation and can limit production of cytokines IL-1β and IL-18 [51]. Induced autophagy to inhibit the inflammasome and excessive inflammation or marking directly specific NLRs (NOD-like

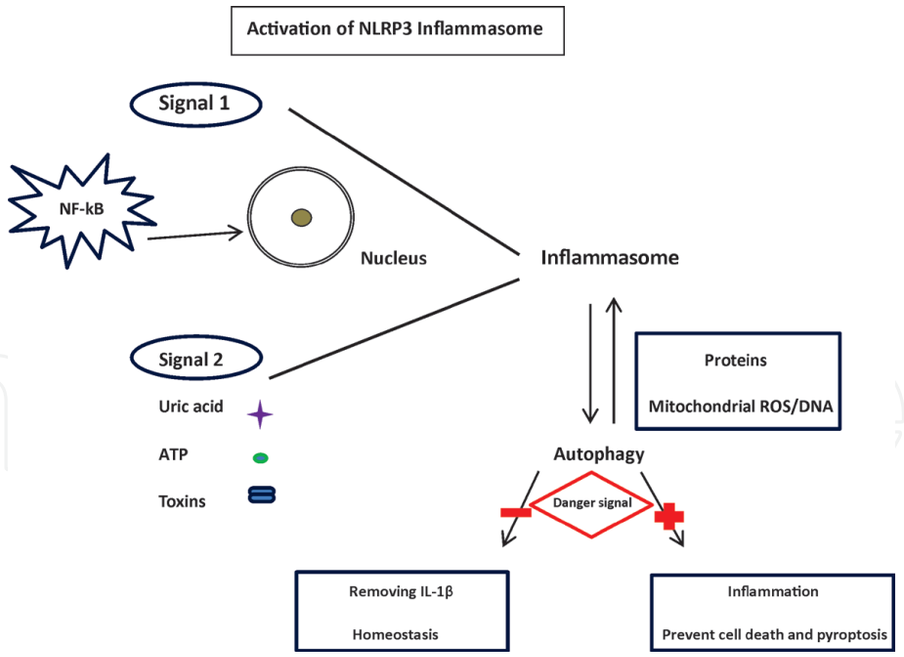

Figure 4.
Activation of NLRP3 inflammasome. This process requires two signals: the first is dependent of NF-kB activators and the second demands ATP, uric acid crystals, bacterial toxins or particulate matter to activate NLRP3 inflammasome. Source: own authorship.

receptors) to reduce its activity may be a promising strategy to reverse inflammatory process [52].

In the present study, the oxidative stress induced by H_2O_2 on placental explants contributed to the inflammatory profile generated by activating the NLRP3 inflammasome, caspase-1 and inducing the release of IL-1β (**Figure 3C** and **D**).

In a previous study published by our research group, we observed that some markers of oxidative stress, such as superoxide dismutase (SOD) and catalase are altered at high concentrations of hydrogen peroxide confirming that H_2O_2 induces oxidative stress on placental explants and demonstrated that this stress involves inflammasome activation [53].

We demonstrated that gene expressions of NLRP3 and caspase-1 have similar patterns, with greater expression in cultures exposed to the concentration of 1000 μM of H_2O_2, which means that this concentration was able to activate the NLRP3 inflammasome (**Figure 5**). The activation of this complex may occur as a

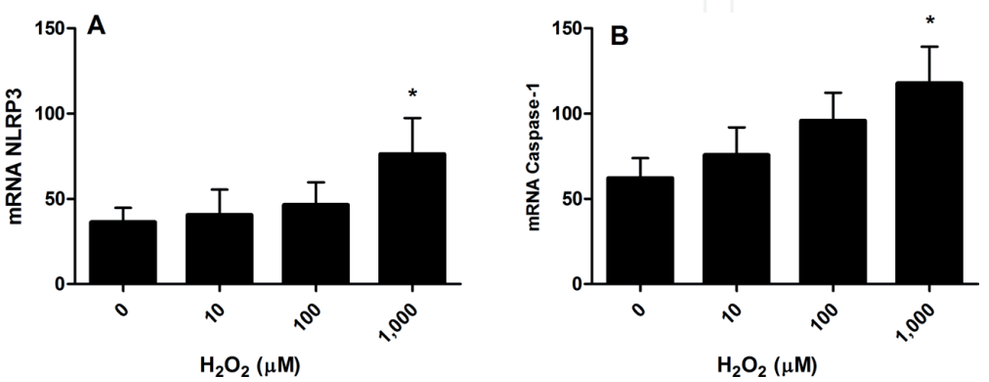

Figure 5.
*Inflammasome activation in placental explants in cultures exposed to different concentrations of hydrogen peroxide. mRNA expression of NLRP3 (A) and caspase-1 (B) in explants of placental explants. Results expressed as mean ± SD. * ($p < 0.05$) vs. 0, 10 μM (ANOVA). Source: own authorship.*

consequence of a common form of cellular stress initiated by different stimuli, such as the release of ROS [54, 55].

The relationship between occurrence of inflammasome and autophagy activation may be explained by the elevation in gene expression of p62 under conditions of oxidative stress. Inflammasome can be degraded by autophagosomes through this protein [56].

4. Conclusion

Taken together, the results of the present study confirm that H_2O_2 induces oxidative stress in placental explants, demonstrated by activation of NLRP3 inflammasome, which in turn induce the autophagy activation in order to control the inflammatory state. Activation of inflammasome and autophagy are essential elements of the innate immune system, and disorders in these processes have been implicated in various inflammatory and infectious diseases [57]. Oxidative stress may also contribute to placental tissue senescence and to the pathophysiology of some placental-related disorders of pregnancy, such as preeclampsia and fetal growth restriction [56]. Thus, initiatives to reduce stress on trophoblastic tissue should be considered for future researches.

Many studies have observed the effects of supplementation to prevent the effects of oxidative stress and autophagy in preeclampsia, such as the use of antioxidants, vitamins C and E, calcium, resveratrol and some natural products [58–61].

The use of natural products and hormones such as Vitamin D may be a new model to reduce inflammation by regulating autophagy, since there is a direct correlation between vitamin D levels and cell survival in pathologies associated with gestation. Vitamin D and its components such as vitamin D receptor (VDR) are molecules that are highly related to the autophagic process [62]. In this sense, the use of products with antioxidant and anti-inflammatory effects still need to be evaluated in order to reduce oxidative stress, induce autophagy, and decrease the activation of inflammasome in placental tissue.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary material

1. Material and methods

1.1 Study population and ethics statement

This study consisted of 15 healthy pregnant women with normal evolution of the pregnancy, with no personal history of hypertensive disorders in pregnancy. These pregnant women were admitted to the Obstetrics Unit of Botucatu Medical School,

Sao Paulo State University, Botucatu, SP, Brazil between November 2015 and May 2016. Gestational age was calculated from the last menstrual period and confirmed by ultrasound dating. Exclusion criteria included chronic hypertension, multiple gestation, prior preeclampsia, illicit drug use, and preexisting medical conditions such as diabetes, cancer, acute infectious disease, cardiovascular, autoimmune, renal and hepatic diseases. The study was approved by the Ethics Committee of the Botucatu Medical School, and written informed consent was obtained from all women involved in the study (CAAE Protocol number: 37160614500005411).

1.2 Collection of placental tissue

All placentas from normotensive pregnant women were delivered by cesarean section, without labor and were examined macroscopically and processed within 10 min of delivery. Fragments of approximately 5 × 5 cm were immediately removed from the central region of the placenta, constituting samples of the villous cytotrophoblast and the syncytiotrophoblast region in contact with the maternal side (basal plate). After collection, the trophoblastic tissue was washed in buffered saline (PBS) and separated from the decidual layer that is normally adhered to the basal plate. The terminal portions of the villi were evidenced in PBS (the villi were seen floating freely in the liquid) and dissected in small sections to constitute explants.

1.3 Culture of placental explants with hydrogen peroxide

The amount of villi used was 11 mg of placental tissue that was cultured in each well of 24-well plates (SPL Life Sciences, Korea) for 24 h for stabilization [63]. Cultures were performed in vitro in the absence of hydrogen peroxide or in the presence of 10, 100, and 1,000 μM of H_2O_2 for 4 h and 24 h in RPMI 1640 culture medium supplemented with 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 40 mg/ml antibiotic/antimycotic (Sigma-Aldrich), and 10% fetal bovine serum (Gibco BRL Life Technologies, The Netherlands) inactivated (complete RPMI medium).

After the culture periods, the explants were removed and submitted to RNA extraction for further analysis of the expression of genes related to inflammation (cytokines), autophagy, and inflammasome. Culture supernatants were obtained, centrifuged at 2,000 g for 10 min and stored at −80°C for determination of cytokines.

1.4 Cell viability assay

The cell viability assay was conducted through the activity of the enzyme lactate dehydrogenase (LDH) in supernatants of placental explant after 24, 48, 72, and 96 h of culture and was determined by commercial kit (Sigma-Aldrich) according to the manufacturer's instructions.

1.5 Evaluation of the expression of transcripts related to inflammation

The placental explants were evaluated for the expression of the genes encoding IL-1β, TNF-α, LC3-II, beclin-1, and p62 proteins at the transcriptional level. In addition, the gene expression of the inflammasome was evaluated through the NLRP3 and caspase-1 genes. Total RNA was extracted from the placentas using the Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada) according to the manufacturer's protocol, and the Reverse Transcription-coupled polymerase chain reaction (RT-qPCR) was performed as described previously [64]. Briefly, isolated RNA was DNAse I Amp Grade (Invitrogen) treated. Subsequently, the synthesis of complementary DNA (cDNA) was conducted using ImProm-IITM Reverse Transcription System,

Gene	Sequence (5′-3′)	Name	GeneBank
Caspase-1	Forward primer: (1065) AGACATCCCACAATGGGCTC(1084) Reverse primer: (1172) TGAAAATCGAACCTTGCGGAAA(1151)	CASP1	NM_033292.3
NLRP3	Forward primer: (2826) GAGGAAAAGGAAGGCCGACA(2845) Reverse primer: (2917) TGGCTGTTCACCAATCCATGA(2897)	NLRP3	NM_004895.4
IL-1β	Forward primer: (544) GAGCAACAAGTGGTGTTCTCC(564) Reverse primer: (653) AACACGCAGGACAGGTACAG(634)	IL1B	NM_000576.2
TNF-α	Forward primer: (325) GCTGCACTTTGGAGTGATCG(344) Reverse primer: (462) GGGTTTGCTACAACATGGGC(443)	TNF	NM_000594.3
p62	Forward primer: (159) CGCTTCAGCTTCTGCTGC(176) Reverse primer: (308) GTCCTCATCGCGGTAGTGC(290)	SQSTM1	NM_003900.4
Beclin-1	Forward primer: (101) GTAGACCGGACTTGGGTGAC(120) Reverse primer: (198) CATGGTGCTGTTGTTGGACG(179)	BECN1	NM_003766.3
LC3-II	Forward primer: (517) CCAGGAAACCTTCGGCTTCT(536) Reverse primer: (632) CGGTAGAGGCAGCTCAGTTC(613)	MP1LC3A	NM_032514.3
GAPDH	Forward primer: (684) CGTGGAAGGACTCATGACCA(703) Reverse primer: (801) GGCAGGGATGATGTTCTGGA(782)	GAPDH	NM_002046.4

Table 1.
Primers for inflammasome and autophagy proteins, cytokines, and GAPDH. Source: Gen Bank (https://www.ncbi.nlm.nih.gov/genbank).

according to manufacturer's protocol. The RT-qPCR was made using RT GoTaq-qPCR Master Mix (Promega, Madison, WI, USA), and the primer sequences used in this study are listed in **Table 1**. Each reaction was set in duplicate and the conditions for the RT-qPCR were as follows: initial denaturation at 96°C for 2 min and then 40 cycles at 95°C for 15 s and 60°C for 60 s, followed by a melting curve. Expression values of the analyzed transcripts were normalized to that of the enzyme-encoding glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH).

The calculation of the differential expression of selected genes was carried out by the data processing method compared with a standard curve [65]. To analyze relative gene expression, we standardized the RNA expression levels in all samples to that of a single RNA sample, which was set to a value of 100.

1.6 Cytokine determination

Cytokine concentrations in culture supernatants of placental explants were determined by enzyme-linked immunosorbent assay (ELISA), using Quantikine

ELISA kits (R&D Systems) for TNF-α and IL-1β according to the manufacturer's instructions. Assay sensitivity limits were 1.6 pg./mL for TNF-α and 3.9 pg./mL for IL-1β.

1.7 Statistical Analysis

All of the data were analyzed using one-way ANOVA, followed by post-hoc Tukey test using the statistical program PRISM (Graph Prism for Windows, version 6.01, GraphPad, EUA) to compare the difference among the groups. A p value less than 0.05 was considered to be statistically significant.

Key points

1. Autophagy is an intracellular degradation system characterized as a natural defense mechanism capable to reduce damages related to inflammatory responses.

2. The main role of autophagy is to maintain cellular homeostasis by recycling intracellular materials.

3. Activation of inflammasome and autophagy are essential elements of the innate immune system, and disorders in these processes have been implicated in various inflammatory and infectious diseases.

4. Oxidative stress may also contribute to placental tissue senescence and to the pathophysiology of some placental-related disorders of pregnancy, such as preeclampsia and fetal growth restriction.

5. The use of products with antioxidant and anti-inflammatory effects still need to be evaluated in order to reduce oxidative stress, induce autophagy, and decrease the activation of inflammasome in placental tissue.

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