

# Antiapoptotic activities of Leukemia Inhibitory Factor (LIF) and Vascular Endothelial Growth Factor (VEGF) in hypoxic human trophoblast depend on gestational age.

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*Abstract:* Susceptibility of the human trophoblast to apoptosis is changing throughout pregnancy. Invasiveness and differentiation of the trophoblastic cells may be severely modulated by hypoxia, well-known apoptosis inducer. From the other hand, local antiapoptotic activities of Leukemia Inhibitory Factor (LIF) and Vascular Endothelial Growth Factor (VEGF) may affect the course of hypoxia-induced programmed cell death. In this study, we examined comparatively (first trimester vs. term pregnancy), whether gestational age as well as LIF- and/or VEGF-dependent changes in functional status of the cultured trophoblast cells influence apoptotic activity in hypoxic conditions. Placental specimens were obtained after abortions in the first trimester (Group I) and after deliveries at term (Group II). The cytotrophoblast cells were isolated using Kliman's method, based on enzymatic digestion and a Percoll gradient centrifugation. Established cultures within both groups were divided into 3 subgroups: LIF- (10ng/mL), VEGF- (100ng/mL) and LIF+VEGF-treated, cultured under hypoxia (2% O<sub>2</sub>) for 48 hours. After this period, quantitative determination of apoptosis was performed using ELISA, with cytokeratin-18 as an apoptosis marker. LIF, VEGF and LIF+VEGF reduced intensity hypoxia-related apoptosis. This effect was stronger in the first-trimester trophoblasts. The differences in mean decrease of apoptosis intensity between Group I and II were significant ( $p < 0.05$ ) and amounted to 76.09, 43.72 and 68.45 % (LIF-, VEGF- and LIF+VEGF-treated cultures, respectively). Protective mechanisms induced by LIF- and VEGF within trophoblast cells may be responsible for the relative resistance to hypoxia and consequently apoptosis, especially in the first-trimester of pregnancy.

*Key-Words:* apoptosis, hypoxia, human trophoblast, leukemia inhibitory factor, vascular endothelial growth factor

## 1 Introduction

Normal placentation and development of the placental vascular network are crucial for adequate blood supply to the fetus [1,2]. In situ formation of blood vessels from progenitor endothelial cells (angioblasts) and development of new blood vessels from pre-existing vasculature play an essential role in embryonic development and normal growth of placental tissue [3]. Both, physiologic and abnormal changes in the local oxygen concentration may significantly influence these processes. Major placental angiogenic factors include the vascular endothelial growth factor (VEGF) gene family of cytokines, which may be up-regulated by decreased oxygen tension [4,5]. Independent authors have reported that hypoxia increases rate of transcription of VEGF and enhances VEGF receptors expression (and probably their affinity to VEGF) in placental membranes, placenta and trophoblast cell

lines [6,7]. Normal course of pregnancy requires precise balance between invasiveness and differentiation. In general, more differentiated cells are less invasive and vice versa [8]. In the human placenta, cytotrophoblasts show two characteristic patterns of differentiation: into syncytiotrophoblast and into extravillous trophoblast [9]. The latter is reported to become invasive and related to proliferation within decidua and maternal spiral arteries in the first trimester of pregnancy. Replacement of the endothelial and muscular linings of the spiral arteries contributes to their widening and increased placental blood flow [10]. It is generally accepted, that extravillous trophoblast cells resemble malignancies with their invasive and destructive features, except that they are sequentially restricted to the first trimester of pregnancy [8]. Trophoblast cells from the term placenta have reduced invasive capacity [11]. Ischemia and hypoxia within the placental tissue may lead to

various complications of pregnancy, including preeclampsia/eclampsia and intrauterine growth retardation (IUGR), [12,13]. A number of factors may contribute to hypoxia-mediated cell death. Hypoxia inhibits oxidative phosphorylation and a switch to glycolytic metabolism, resulting in decreased concentrations of energy-rich phosphates (ATPs), overproduction of lactic acid, and consequently - lowered intracellular pH [14]. Rapidly decreased ATPs levels produce necrosis with passive loss of transmembrane ion gradients, followed by cell swelling and loss of membrane integrity. Unlike necrosis, apoptosis is a process in which membrane integrity is preserved until very late stages. Moreover, programmed cell death is an active, energy-consuming process that must be initiated in the presence of sufficient cellular ATPs [15]. Modulation of pro- and anti-apoptotic factors may be involved in important homeostatic mechanisms that enable trophoblast cells to survive. As the properties of human trophoblast change over the course of pregnancy (e.g., susceptibility to hypoxia and hypoxia-related apoptotic triggers), it has been suggested that the hypoxic environment may be to some extent physiologic for the first trimester trophoblastic cells, but abnormal and affecting fetal well-being in the later trimesters [12,16, 17].

Placental villous hypoxia reduces trophoblast differentiation and increases apoptosis in the trophoblast cells [16,18]. As one of the main forms of programmed cell death in multicellular organisms, apoptosis plays an important role in the physiology and pathophysiology of the human trophoblast [19]. This very complex process is a component of normal development and differentiation within placental tissue and includes a cascade of events involving the activation of many genes and the production of various protein-based compounds [20,21]. Typically, apoptosis is followed by increased enzymatic activity. Caspases are a group of cysteine proteases critical for apoptosis of eukaryotic cells. Data accumulated after deletion of genes that encode caspases suggests that caspases are involved not only in apoptosis but also in cytokine maturation and cell growth and differentiation [22]. Caspases exist as latent zymogens that contain an N-terminal pro-domain followed by the region that forms a two-subunit catalytic effector domain. Despite of similarities in amino acid sequence and structure, the members of caspase family differ in their physiologic properties [23]. According to these differences, it is reasonable to divide the caspase family into two groups or classes. The first group includes caspases that are centrally involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10), and the second group - those related to caspase-1 (caspase-1, -4, -5, -13, and -14, as

well as murine caspase-11 and -12), whose are primarily involved in the processing of pro-inflammatory cytokines [24].

It was suggested in many publications pertaining to molecular and biological aspects of cell physiology, that caspases function in cell-to-cell signaling processes, including apoptosis, cell growth and differentiation. However, alteration of the precise balance between cellular proliferation, differentiation and death is considered a crucial component in cytotrophoblast-related placental dysfunction [19,25,26]. There are many factors that may affect apoptosis, including leukemia inhibitory factor (LIF) and VEGFs [27,28]. LIF, an interleukin 6 class pleiotropic cytokine, binds to the specific LIF receptor (LIFR- $\alpha$ ), which forms a heterodimer with a specific subunit (GP130 signal transducing subunit) common to all members of that family of receptors [27]. Although the inhibition of myeloid leukemic cell lines through the induction of differentiation is a well known ability of LIF (defining its name), this cytokine exerts different biological actions in various tissue systems. Data about the role of LIF in reproduction are still accumulating. Song & Lim demonstrated that LIF is crucial for the successful implantation of embryos in mice [29].

Both LIF and VEGF influence trophoblast invasiveness and differentiation through the inhibition of apoptosis, acting via the Janus kinase (JAK)/Signal Transducers and Activators of Transcription (STATs) pathway [30]. STAT3 and STAT5 induce growth (including tumor progression) through the cell cycle, prevent apoptosis, and upregulate oncogenes such as c-myc and bcl-X [31]. LIF, a member of the IL-6 family of cytokines, signals via STAT3. VEGF uses STAT3 and STAT5 for antiapoptotic signaling, impinging on the transcription of FAS, a well-known proapoptotic gene [27,32]. VEGF activates STATs via VEGF receptor-2 (KDR) [33]. It has been recently suggested that the VEGF/JAK2/STAT5 signaling axis may partially mediate tolerance to cell hypoxia [32]. In independent studies, the expression of LIF and VEGF as well as the respective receptors have been confirmed in both human cytotrophoblast cells and in vitro differentiated syncytiotrophoblast cells [27,34].

## 2 Aim of the study

The aim of the present study was to compare first trimester and third trimester human trophoblast cells to ascertain whether LIF- and/or VEGF-dependent changes in the functional status of cultured in vitro trophoblast cells influence apoptotic susceptibility under hypoxic conditions.

### 3 Materials and methods

This study was conducted in compliance with international and local laws of human experimentation and the project was approved by the local ethics committee. All subjects provided written ethical consent to the collection and use of their tissues, according to the standards published by British Medical Research Council as "Human tissue and use of biological samples: operational and ethical guidance 2001 (includes Addendum to MRC 2001 guidance following the Human Tissue Act 2004)". All data and records were properly created, maintained and stored by authorized research personnel for this study.

#### 3.1 Placental sample collection

First trimester placental tissue samples (approx. 1.5 x 1.5 x 1.0 cm) were obtained within 60 min after spontaneous missed abortions of unknown origin (without any recognized underlying disease, excluding cases with evidence of an inflammatory process) between the tenth and the twelfth week of gestation (Group I; N=16; mean gestational age  $76 \pm 6$  days). Time of onset of abortion was determined by absence of the fetal heart rate (FHR) in repeated ultrasonographic examination performed at hourly intervals. Third trimester placental tissue samples were obtained after normal term pregnancies (Group II; N=16; mean gestational age  $273 \pm 6$  days). From the maternal surface of each placenta, three samples were excised in a standardized manner (the first one – from the central part, and the next two – from peripheral regions contiguous to this maternal surface; see Fig.1.); therefore 48 trophoblast cultures were developed immediately after the samples collection.

#### 3.2 Trophoblast cultures

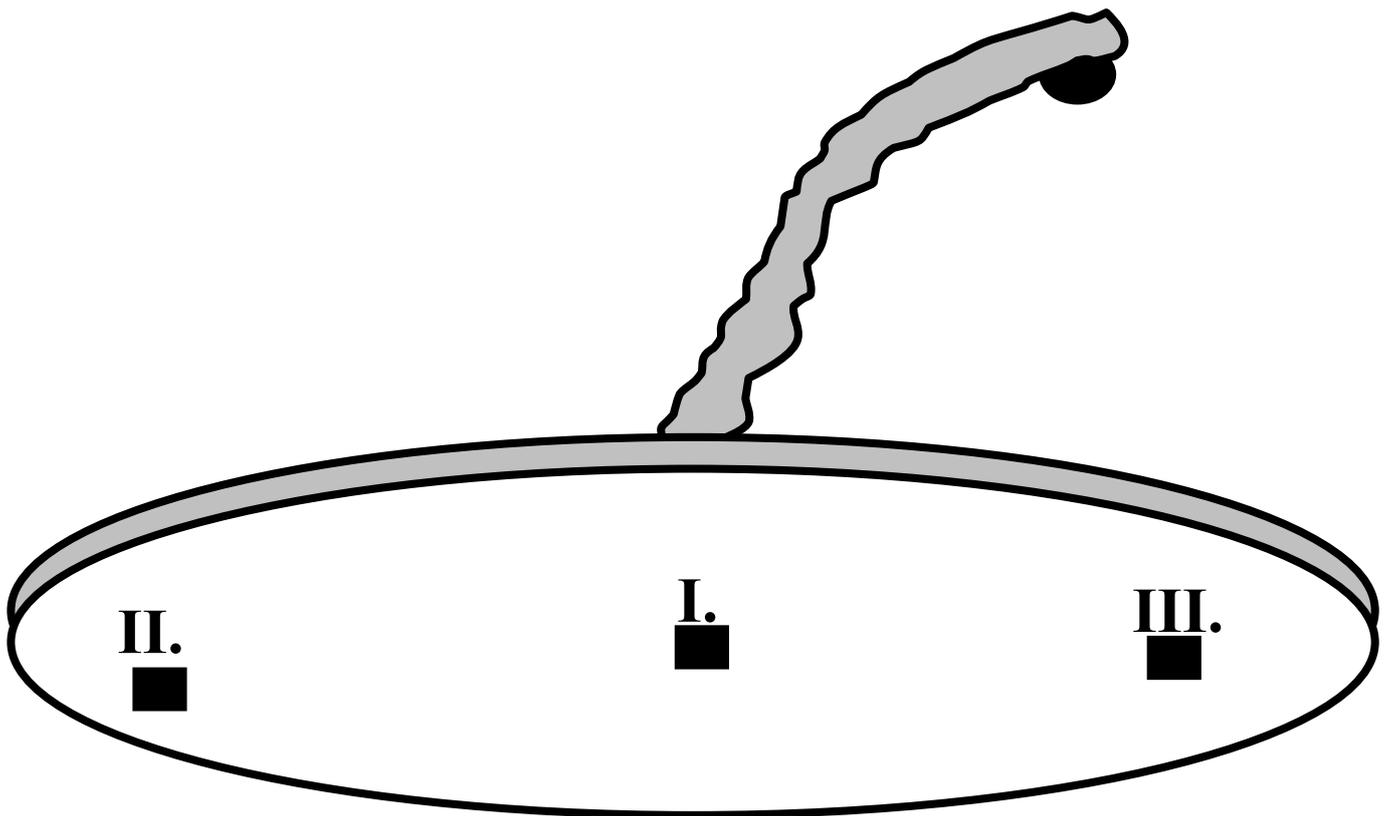
The cytotrophoblast cells were isolated from villous tissue using our modification of the method given previously by Kliman *et al.* [35]. This method has been described in detail elsewhere [36,37]. Briefly, placental specimens were rinsed with saline to remove blood cells. Minced villous tissue was digested with 0.125% trypsin (Sigma Chemical Co., St. Louis, MO, USA) and 0.2 mg/mL deoxyribonuclease I for 30 min at 37 degrees C. Following enzymatic digestion, the resultant cell suspension was filtered, dispersed and fractionated by centrifugation through a 5-70% Percoll gradient (1200 rpm for 20 min). The cell layers that accumulated between 40-50% (density 1.048-1.060 g/mL) were collected, washed and resuspended ( $5 \times 10^5$  cells/ml) in Ham's F12/Dulbecco's modified Eagle's medium (1:1) with 15% fetal bovine serum. This procedure yields a highly purified preparation

(approx. 95% pure) of cytotrophoblast cells with >90% viability, determined by phase contrast microscopy. After 5 days, the cultures within both groups were divided into three subgroups: LIF- (10ng/mL), VEGF- (100ng/mL) and LIF+VEGF-treated, then cultured under hypoxic conditions (2% O<sub>2</sub>). After 48 hours, the cultures were terminated and apoptosis intensity measurements were performed. Respective normoxic and hypoxic controls were also established.

#### 3.3 Assessment of apoptosis intensity

During apoptosis, caspases cleave various cellular proteins. In the cells of epithelial (endodermal) origin, the intermediate filament cytokeratin 18 represents (M30 neo-epitope) one of the major caspase substrates and is present in abundant concentrations. As mentioned substrate is also released from apoptotic trophoblasts, the cell culture supernatants do contain M30 neo-epitope. Thus, quantitative determination of apoptosis was performed using an immunoenzymatic assay with antibodies against a neo-epitope of cytokeratin 18 (an apoptosis marker) [38].

The culture supernatants were stored at 4°C no longer than 24 hours before the beginning of apoptosis assessment. M30-Apoptosense (Peviva AB, Sweden) was used for specific and sensitive detection of the caspase-cleaved intermediate filament protein cytokeratin-18. The assay is a solid-phase, two-site immunosorbent assay (ELISA). Samples (25 µl) containing caspase-cleaved CK18 (CK18Asp396-NE: M30 neo-epitope) added into the wells bind to an immobilized monoclonal catcher antibody specific to CK18 and are subsequently detected with the CK18Asp396-NE-specific M30 monoclonal antibody conjugated to HRP as a tracer. The M30 antibody is a mouse monoclonal of the IgG2b subtype that recognizes a neo-epitope in the C-terminal domain of cytokeratin-18 (amino acids 387-396: CK18 Asp396-NE), which is exposed after cleavage by multiple caspases during apoptosis. Caspases that are capable of cleaving CK 18 to generate this M30 neo-epitope include in addition to caspase-3, also caspase-6, -7, and -9. After the formation of the solid phase/antigen/labeled antibody sandwich, excess unbound tracer is removed by washing. Tetramethylbenzidine (TMB) substrate is then added, and the reaction is stopped after a defined incubation period. The absorbance is measured in a microplate reader at 450 nm. By plotting a standard curve from known concentrations versus measured absorbance, the amount of M30 antigen present in the sample can be calculated. The concentration of the M30 antigen is expressed as units per liter (U/L).



**Figure 1.**

Location of samples (I-III) collected from the maternal surface of the placenta in standardized manner. The mean weight of samples  $10.53 \pm 0.95\text{g}$ .

### 3.4 Statistical analysis

The results are reported as mean percentage values  $\pm$ SEM of compared control values (taken as 100% for Group II). Completed data were subjected to statistical analyses in order to determine whether observed differences in the mean percentages of apoptotic intensity are significant. Mann-Whitney's U test was applied, and the significance threshold was  $p < 0.05$ .

## 4. Results

The results are presented as bar graphs in Fig. 2. In both groups, administration of LIF, VEGF and LIF+VEGF produced a significant ( $p < 0.05$ ) decrease in hypoxia-related apoptosis. During hypoxia, this anti-apoptotic influence was stronger in the first-trimester trophoblasts. As shown in Fig. 2A, the differences in the mean reduction of apoptosis intensity between Group I and Group II amounted to

76.09%, 43.72% and 68.45% (LIF-, VEGF- and LIF+VEGF-treated cultures, respectively;  $p < 0.05$ ).

The results obtained in LIF+VEGF treated, the first-trimester trophoblast hypoxic subgroup (not substantially different from both, LIF- and VEGF-treated the respective subgroups) may suggest that LIF and VEGF at least partially compete for the same signaling pathway.

In hypoxic control cultures without exogenous LIF or VEGF, the differences in apoptosis intensity between first-trimester trophoblasts and term trophoblasts were not significant (see Fig. 2A Control).

In normoxia, the addition of LIF, VEGF or LIF+VEGF to the culture media also diminished the mean intensity of apoptosis as compared to controls (see Fig. 2B), but this effect was similar in both studied groups ( $p > 0.05$ ). Apoptotic intensity in normoxic controls (Fig. 2B Control) was significantly

lower than in the hypoxic controls. The concentration of the caspase-cleaved intermediate filament protein cytokeratin-18 fragments (CK18Asp396-NE) in the culture supernatants ranged from 122 U/L to 321 U/L (normoxic versus hypoxic controls, respectively).

## 5. Discussion

First suggestion about different, gestational age-dependent susceptibility of the hypoxic human trophoblast to apoptosis was given in our previous preliminary report [39]. Now, we analyzed the results more thoroughly.

It should be noticed, that still evolving apoptosis detection methodology may considerably influence the results. Some methods, such as TUNEL (labeling of double-stranded DNA breaks) do not distinguish between necrotic and apoptotic cells. Considering, that the caspase family of cysteine proteases plays a central role in this environmentally conserved mechanism of regulated cell death, we used The M30 CytoDeath™ ELISA. Due to the specificity of the M30 antibody to a caspase cleavage product, the M30-Apoptosense® assay recognizes apoptotic cells only, does not react with viable or necrotic cells. Apoptosis detection with the M30 CytoDEATH assay has been described in several publications as a superior quantitative technique when compared to TUNEL or to detection of gammaH2AX phosphorylation [40]. For example, the M30-Apoptosense® assay detects apoptosis early on, as opposed to methods that measure DNA fragmentation.

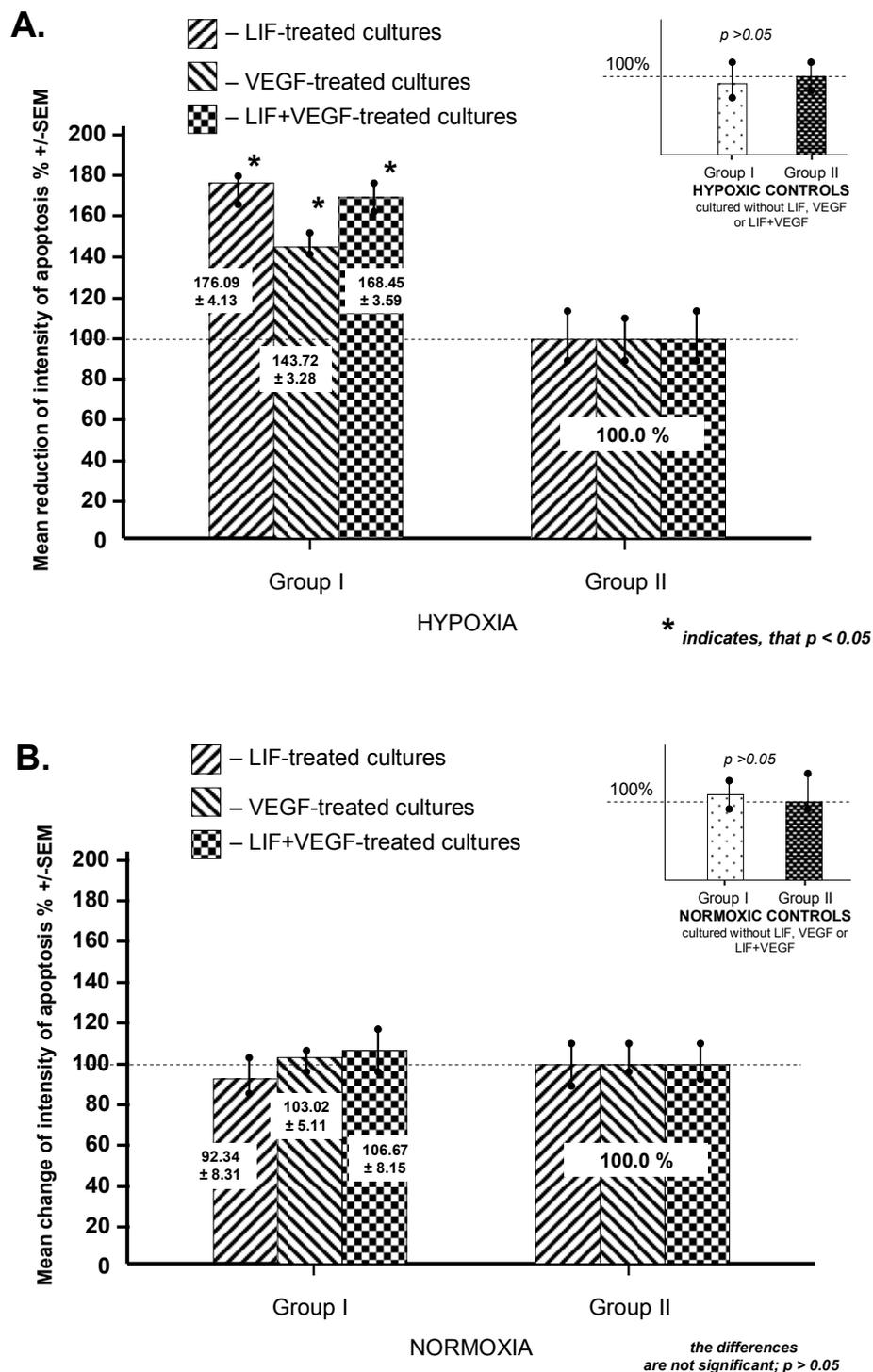
Apoptosis was originally defined by Kerr *et al.*, in their well-known, highly cited paper, published in 1972 [41]. The definition of this distinct form of cell death associated with normal physiology was based purely on cell's morphology. Apoptosis was distinguished from necrosis, which is associated with acute injury to cells. Apoptosis is characterized by nuclear chromatin condensation, cell shrinking, dilated endoplasmic reticulum, and membrane blebbing [41]. Consequently, the term apoptosis has been applied to anything that looks like apoptosis.

Despite the widespread use of the apoptosis-versus-necrosis paradigm, there is an increasing awareness of the complexity of processes occurring in dying cells that lead to the outcome of death. Recent experimental data have led to significant advances impacting on our understanding of biochemical mechanisms responsible for changes in cell morphology [23]. Considering this, it has been proposed that cell death resulting from intrinsic cellular processes should be interpreted as distinctly different from cell death caused by severe environmental perturbations [24]. To simplify, Samali

*et al.* have proposed that apoptosis should be defined as caspase-mediated cell death [42]. Another apoptosis look-like phenomenon has been distinguished and described independently [43,44,45]. The term "pyroptosis" or "caspase-1-dependent form of programmed cell death" was proposed. Pyroptosis is inherently inflammatory and associated with antimicrobial responses. The mechanisms and outcome of pyroptosis are therefore distinctly different from observed during apoptosis, which actively inhibits inflammation.

Even if pyroptosis is related to increased levels of caspase cleavage products reacting with antibodies against a neo-epitope of cytokeratin 18, in our study we avoided as much as possible infected trophoblast cells, both during collection of placental samples and in vitro culture. Apoptosis occurs in most tissues as an important process related to normal development and differentiation [46]. Within trophoblast cells of the human placenta, disturbances of the ratios between cellular growth/differentiation and programmed cell death may produce abnormal placental function [19,25,26]. Hypoxia is known as a deteriorating factor in most tissues, acting as an inducer of apoptosis [26]. On the other hand, properties of trophoblast cells are very unique, because early placental development occurs in an environment of relative oxygen deficiency [3,12]. The relative resistance of early trophoblast cells (transforming into the syncytiotrophoblast phenotype) to acute and extreme hypoxia may be a defense mechanism by which this crucial cell layer preserves critical functions even in the presence of extreme environmental stress. Tolerance to hypoxia may be closely related with the type of JAK/STAT pathway activation [32]. For instance, in cardiomyocytes undergoing hypoxia-reperfusion injury, STAT1 propagates a pro-apoptotic signal, whereas STAT5 seems to play the opposite role, activating antiapoptotic survival signals [47]. Constitutively active STAT3 is present in many malignancies and acting as an oncogene by inhibition of apoptosis, even during hypoxia [48, 49]. Invasive properties of first-trimester trophoblast may be augmented by several members of the IL-6 family of cytokines acting via STAT3, including LIF [27]. Thus, decreased antiapoptotic effects of LIF in term trophoblast parallel its reduced invasiveness.

Hypoxia promotes angiogenesis, increases local VEGF concentration and up-regulates the expression of type 1 and 2 VEGF receptors [12,16,36]. Hypoxia is a broad term meaning diminished availability of oxygen (in experimental tissue culture settings in vitro, usually the level of oxygen is kept within the range 2-5%) and should not be confused with anoxia, which means a total lack of oxygen.



**Figure 2.**

Influence of LIF, VEGF and LIF+VEGF on the mean reduction/change of apoptosis intensity in hypoxia and normoxia (A. & B., respectively): first-trimester (Group I) versus term (Group II) trophoblast cultured in vitro. The mean intensity of apoptosis for term trophoblast was taken as 100%.

It is very likely that sensitivity of the human trophoblast to apoptosis changes throughout normal pregnancy, but may be disturbed in abnormal hypoxic conditions. Proteomic analyses of mechanisms of hypoxia-induced apoptosis in trophoblast cells have revealed many potential markers of placental hypoxia-related obstetrics complications, including pre-eclampsia and IUGR [50]. However, usage of the choriocarcinoma cell line JAR by the authors of this interesting study, instead of placental trophoblasts from different trimesters will produce many difficulties in clinical implications of the results.

In many obstetric pathologies characterized by placental underperfusion and villous hypoxia e.g., pregnancy induced hypertension, anemia, intrauterine growth retardation (IUGR), diabetic pregnancy, cigarette smoking and living in a high altitude, increased apoptosis has been reported [13,25,51]. On the other hand, Khaliq *et al.*[52] suggested "placental hyperoxia" instead of "placental hypoxia" as the crucial factor involved in the pathophysiology of IUGR. Apoptosis in tissues is induced by different kind of signals including endogenous aldehydes. The accumulation rate of aldehydes in the cell is affected by conditions of oxidative stress [53]. It is generally accepted that the fetoplacental unit is exposed to oxidative stress and that in certain conditions the placental cells including trophoblasts are very susceptible to peroxidation [54]. Therefore, the efficiency of enzymatic (including various isoforms of aldehyde dehydrogenase, aldehyde reductase, and glutathione-S-transferase) and non-enzymatic reactive oxygen scavengers must be ensured throughout pregnancy for normal fetal growth and development, not only in early but also in late pregnancy [55]. There is evidence suggesting that the catalytic properties of these enzymes change during implantation and placental development, and that aging of the trophoblast may be accompanied by their reduced activities [54,55]. The role of malondialdehyde accumulation and alteration of aldehyde's scavenger enzymes should be considered as apoptosis-promoting factors, especially in the third trimester or term trophoblast [54,56]. Both, physiologic and disease-influenced responses of the human trophoblast to these apoptotic stimuli may be crucial as compensatory mechanisms or the reasons for placental insufficiency. Accumulating data also suggest that complex process of villous development and maturity might be influenced by the maternal and fetal oxidative and angiogenic impairments [3,16,57].

Interestingly, both hypoxia and hyperoxia promote programmed cell death [16,58].

Because of their clinical importance, the molecular and cellular mechanisms of the trophoblast cells

responses to hypoxia have been studied extensively in recent years [12,16,19]. Oxygen deficiency below a certain level is known to hinder differentiation in cultured trophoblasts, while enhancing the expression of p53 and Bax, two proapoptotic proteins that predominate in cytotrophoblast [16,19,59]. The differentiated trophoblast phenotype (i.e., syncytiotrophoblast) is more resistant to hypoxia-related apoptosis than the less differentiated cytotrophoblast phenotype. Levy *et al.*[60] found that programmed cell death occurred primarily in the first 24 hours of culture, when most of the cells are undifferentiated cytotrophoblasts [61]. Thus, we hypothesized that the greater resistance of the first trimester trophoblasts to apoptotic stimuli under hypoxia may be related to their ability to differentiate more quickly than the third trimester cultured trophoblast, governed by LIF and VEGF. Further experiments are needed to test this hypothesis. James *et al.* [62] reported recently that both the week of gestation (i.e., gestational age of the trophoblast) and oxygen concentration play important regulatory roles in extravillous trophoblast outgrowth formation during the first trimester. In our study, we analyzed the influence of hypoxia on trophoblastic cells derived from two more distant time points in pregnancy. To the best of our knowledge, this is the first full-length paper to analyze the importance of gestational age of human trophoblasts with respect to LIF and VEGF antiapoptotic activities.

It is important to note that we cultured highly purified trophoblasts, avoiding the influence of other cells and related factors normally present in placental trophoblast surroundings. For example, in vivo the role of placental mast cells (MC) in trophoblast differentiation and apoptosis should be taken into consideration. Human placenta is a relatively rich source of MC, containing both VEGF and LIF as well as many other substances in their vesicular structures [63,64,65]. Moreover, we reported recently that histamine, a major MC amine mediator, is involved in the human trophoblast differentiation, increasing this process by the stimulation of  $\alpha$ - $\beta$ 3 integrins through the histamine H1 receptor [66]. Previously, we found that MC numbers and the concentration of histamine are different in human placental tissue obtained after normal versus complicated pregnancies [67,68]. This may be particularly important considering that hypoxia promotes degranulation of MC and thus the release of many (both pro- and antiapoptotic) mediators [69].

Interestingly, in another study of human term trophoblasts co-cultured with MC, we observed that MC-derived VEGF diminished the expression of the VEGF type 2 receptor (KDR) during hypoxia [36]. Thus, under certain conditions, significant alteration of the VEGF/JAK2/STAT5 axis may reduce the hypoxic

tolerance level of trophoblast cells, leading to increased apoptosis [32].

We realize that our study has some limitations in that our first trimester human trophoblasts were obtained after spontaneous abortions. For example, pathologic conditions caused by autoantibody production may contribute significantly to immune-related-abortions [70,71]. It has been reported that in many diseases autoimmunity is at least partially accompanied by shifted (typically: down-regulated) apoptotic activity within affected tissue [72,73,74]. However, inclusion/exclusion criteria in our study avoided collection of the trophoblast specimens from patients with history of habitual abortion and positive for anti-phosphatidylserine antibodies. These antibodies are thought to be the most significant marker with respect to pregnancy loss [71]. Nevertheless, the results pertaining to the first trimester trophoblasts should be confirmed in further studies conducted with first trimester trophoblast cultures established after elective surgical termination of pregnancy.

## 5. Conclusion

On the basis of our results and considering published data of another, independent authors, we can hypothesize that LIF- and VEGF-related increases in the differentiation and invasiveness of trophoblast cells may protect the cells during hypoxia and consequently reduce apoptosis intensity, especially during the first trimester of pregnancy.

New methods that allow for the improved detection and monitoring of the local hypoxia/hyperoxia in vivo and the apoptosis-associated proteases are key for further advancement of our understanding of apoptosis-mediated disease states such as habitual abortion, placental insufficiency, as well as malignancies and Alzheimer's disease [75].

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