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Modulation of Bax Expression in Physiological and Pathological Human Placentas Throughout Pregnancy

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Abstract. *Apoptosis is intimately involved in placental homeostasis, growth and remodelling, and apoptotic rates increase progressively during normal pregnancy as part of normal placental development. Moreover, apoptosis increases in pregnancies complicated by some pathologies such as preeclampsia, fetal growth restriction and diabetes. In the present study, we describe differences in the expression of pro-apoptotic protein Bax, in first trimester voluntary termination of pregnancy, first trimester abortion (reserved abortion), caesarean birth, spontaneous birth, preeclampsia and diabetes. We first observed a strong increase of Bax expression in the cytotrophoblast, stroma, endothelial cells and decidua of placentas of the first trimester abortion compared to the low/moderate Bax immunopositivity in all the placental compartments during the first trimester voluntary termination of pregnancy. Secondly, we showed a more intense immunopositivity for Bax in the third trimester spontaneous birth with respect to the third trimester caesarean birth. Thirdly, we observed an increase of Bax expression in preeclamptic placentas compared to the normal full-term placentas. In contrast, we observed a moderate Bax expression in diabetic placentas only slightly lower than the normal full-term placentas. Our results seem to suggest that deregulation of apoptotic turnover may lead to placental dysfunction and pathologies.*

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The human placenta is formed by an inner layer of mononucleated trophoblasts, called the cytotrophoblast, that surrounds the blastocoel (1). On direct contact with maternal tissues, the cytotrophoblast fuses to form an outer layer of postmitotic multinucleated cells, called the syncytiotrophoblast (1, 2), which grows by continued incorporation of new mononucleated trophoblasts from a proximal subset of stem cells (3). Some mononucleated cytotrophoblast cells break through the syncytiotrophoblast and invade the uterine stroma forming the trophoblastic cell columns; such cells are called extravillous trophoblasts (EVT) (1). At least two main subpopulations of EVT exist: a) interstitial trophoblast, comprising all those EVT that invade uterine tissues and that are not located inside vessel walls; b) endovascular trophoblast, located inside the media or lining the spiral artery lumina (1). It has been demonstrated that the process of syncytial fusion is linked to the early stages of the apoptosis cascade inside the cytotrophoblast. Since the fusion progress integrates the cytoplasmic content and nuclei from the cytotrophoblast into the syncytiotrophoblast, this process supplements the syncytiotrophoblast with the complete machinery of the apoptosis cascade into the outer layer of placental villi (1). These proteins include antiapoptotic enzymes, explaining why the apoptosis cascade does not immediately progress to its final events inside the syncytiotrophoblast (1). In contrast, the extrusion of syncytial knots from the syncytiotrophoblast is due to the final execution stages of the apoptosis cascade inside the outer placental layer (1, 4-8).

At present, the mechanisms through which the apoptosis cascade is initiated in the cytotrophoblast and then subsequently regulated in the syncytiotrophoblast, remain still unclear. However, several authors have demonstrated that the apoptotic rate increases progressively during normal gestation, being interpreted as part of normal

placental development (9-12). The Bcl-2 protein family is one of the main groups of molecules that play a significant role in the regulation of apoptosis (13). Some proteins from this family, including Bcl-2, inhibit programmed cell death, while others, like Bax, promote it. Complex formation among anti- and pro-apoptotic proteins seems to regulate cellular sensitivity to apoptosis (9, 14-17). An abnormal level of apoptosis also has been correlated with a great variety of gestational pathologies such as placental abortions, ectopic pregnancy, intrauterine growth retardation, post-term pregnancy, preeclampsia and diabetes (10, 12, 13, 18, 19).

In the present study, we describe differences in the expression of Bax in first trimester voluntary termination of pregnancy, first trimester abortion (reserved abortion), caesarean birth, spontaneous birth, preeclampsia and diabetes.

Materials and Methods

Samples. Human placental samples were obtained with informed consent from patients undergoing surgery: as first trimester voluntary termination of pregnancy (n=15), first trimester abortion (reserved abortion) (n=15), caesarean birth (n=15), spontaneous birth (n=15), preeclampsia (n=15) and diabetes (n=15). The gestation period ranged from 5 to 40 weeks. The specimens were immediately fixed in formalin for immunohistochemistry.

Immunohistochemistry. Immunohistochemistry was carried out essentially as described elsewhere (20, 21). Briefly, paraffin-embedded sections from each specimen were cut at 5 μm, mounted on glass and dried overnight at 37˚C. All sections were then deparaffinized in xylene, rehydrated through a graded series of alcohol and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS-6% non-fat dry milk (Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. Slides were then incubated at 4˚C overnight with monoclonal antibody raised against Bax (mouse monoclonal, sc-7480 from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution. After several washes (3x5 min) to remove excess antibody, the slides were incubated with diluted anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. DAB (Vector Laboratories) was used as the chromogen and hematoxylin was used as nuclear counterstain. Negative controls for each tissue section were prepared by substituting the primary antiserum with non-immune IgG. For each experiment, all slides were stained in a single batch and thus received equal staining. Immunohistochemical staining intensity was evaluated by ranking: 0 (absent), 1 (weak), 2 (moderate) or 3 (intense) as described by Seelam *et al.* (22). For each specimen, an HSCORE value was derived by summing the percentages of cells/areas that stained at each intensity and multiplying that by the weighted intensity of the staining. For example: HSCORE = $\Sigma P_i (i + 1)$ where i represents the intensity scores and P_i the corresponding percentage of cells/areas.

An average of 22 fields were observed for each tissue by three observers at different times and the average score was used. All values were expressed as mean ± standard error of mean (SEM) and differences were compared using Student's *t*-test.

Classification of local subtypes of EVT. EVTs were classified as proposed by Kaufmann and Castellucci (23) into two morphological phenotypes: a) the proliferative phenotype, represented by one to several compact layers of cells which are attached to each other; b) the invasive phenotype, comprising the later post-proliferative EVT that no longer forms intracellular junctions and become separated from each other and spread from the cell column into the surrounding basal plate and into the endometrium. The two different phenotypes can easily be discriminated by their topographical relations.

Results

Expression of Bax in the human placenta of the first trimester of gestation. We investigated the localization and distribution of Bax in the first trimester voluntary termination of pregnancy and in the first trimester abortion (reserved abortion) by immunohistochemistry. In the first trimester voluntary termination of gestation (VTG), Bax was localized in the cytoplasm of cytotrophoblast (Figure 1 a) and in the cytoplasm of decidua at a moderate level of expression. In contrast, the Bax immunopositivity in the syncytiotrophoblast was faint. Moreover, Bax had a low immunopositivity in the stroma, in the cytoplasm of endothelial cells and of extravillous trophoblasts (EVTs) (Figure 1 b). In the first trimester abortions (reserved abortions), we observed an intense immunopositivity for Bax in the cytoplasm of cytotrophoblast cells (Figure 1 c), in the cytoplasm of decidual cells, other than in the stroma and endothelial cells (Figure 1 d).

In Figure 2, the expression pattern of Bax immuno-positivity in the first trimester voluntary termination of pregnancy and in the first trimester abortion, as detected by immunohistochemical staining intensity analysis, is compared. A strong increase of Bax expression was found in the cytotrophoblast, stroma, endothelial cells and decidua of placentas of the first trimester abortion compared to the low/moderate Bax immunopositivity in the placental compartment (cytotrophoblast, stroma, endothelial cells, decidua and EVTs) of the first trimester voluntary termination of pregnancy.

Expression of Bax in the human placenta of the third trimester of gestation. We compared the localization and the expression of Bax among third trimester spontaneous birth and caesarean birth using immunohistochemistry. In the third trimester spontaneous birth, Bax had an intense immunopositivity both in the cytotrophoblast and syncytiotrophoblast, in the EVTs and decidua (Figure 3 a, b). In the third trimester caesarean birth, a moderate immunopositivity for Bax was found in the

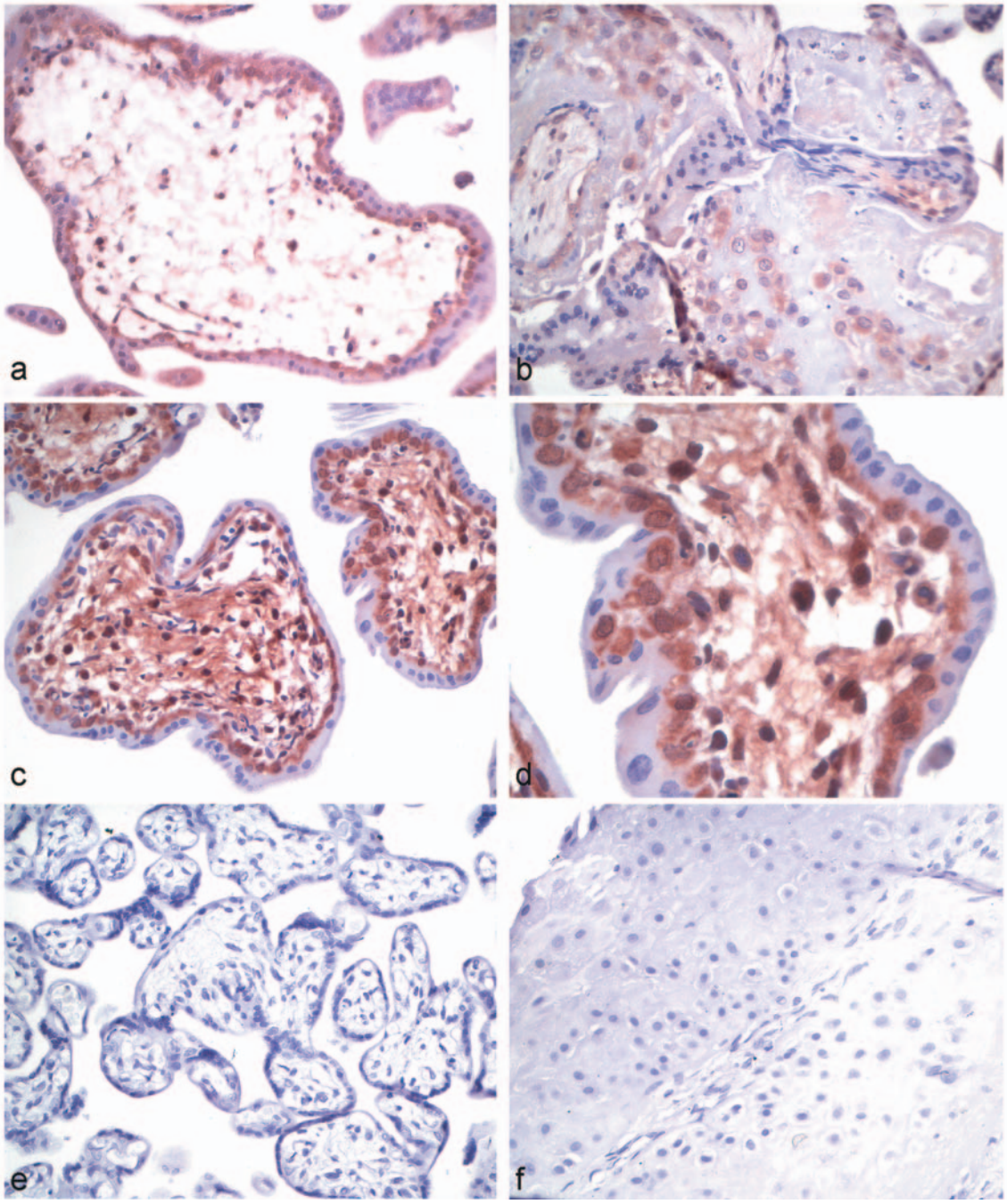

Figure 1. *Localization of Bax in human placenta of the first trimester of gestation. a) Bax immunopositivity in the placenta of first trimester voluntary termination of pregnancy, x150; b) Low immunopositivity in extravillous trophoblasts (EVTs) of the placenta of first trimester voluntary termination of pregnancy, x150; c) Bax immunopositivity in the placental villous of the first trimester abortion, x150; d) Intense Bax immunolocalization in the cytotrophoblast, stroma and endothelial cells of the first trimester abortion placental villous, x300; e) Representative negative control of a placenta of the first trimester of gestation, x100; f) Representative negative showing EVTs, x150.*

cytotrophoblast, stroma, endothelial cells, together with a moderate Bax immunopositivity in the EVTs (Figure 3 c, d) was observed. Only some syncytiotrophoblast showed a weak immunopositivity for Bax.

In Figure 4, the expression pattern of Bax immunopositivity in the third trimester spontaneous birth and caesarean, as detected by immunohistochemical staining intensity analysis, is compared. A more intense

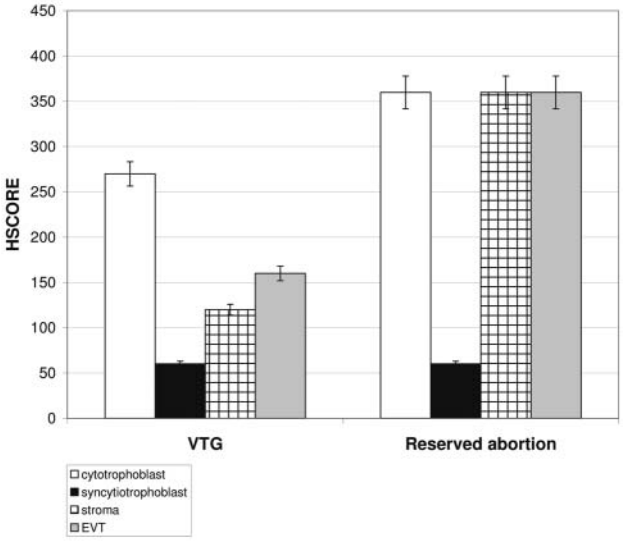

Figure 2. *Expression pattern of Bax immunopositivity in the first trimester voluntary termination of pregnancy and in the first trimester abortion. Vertical lines show S.E.M.*

immunopositivity for Bax was found in the third trimester spontaneous birth with respect to the third trimester caesarean birth, where only the EVTs showed an intense Bax immunostaining.

Expression of Bax in placentas of preeclamptic and diabetic patients in the third trimester of gestation. We investigated the localization and the expression of Bax in placentas of preeclamptic and diabetic patients in the third trimester of gestation by immunohistochemistry. In preeclamptic patients, Bax had an intense immunopositivity in all the placental compartments (cytotrophoblast, syncytiotrophoblast, stroma and endothelial cells), together with a moderate immunopositivity in the EVTs (Figure 5 a). In placenta from diabetic patients, a moderate immunopositivity for Bax was seen in the cytoplasm of cytotrophoblast, stroma, endothelial cells, together with an intense Bax immunopositivity in the EVTs (Figure 5 b). Immunopositivity in the syncytiotro-phoblast was weak.

In Figure 6, the expression pattern of Bax immunopositivity in placentas from preeclamptic and diabetic patients in the third trimester of gestation, as detected by immunohistochemical staining intensity analysis, is compared to the third trimester caesarean birth. A more intense immunopositivity for Bax was observed in almost all the placental compartments of preeclamptic patients with respect to that from placentas obtained by caesarean birth. In contrast, in placentas from diabetic patients only immunopositivity of EVTs was much higher with respect to that of caesarean birth.

Discussion

Programmed cell death by apoptosis and its associated regulatory mechanisms are intimately involved in placental homeostasis, growth and remodeling (13, 24, 25). Several studies have demonstrated that the apoptotic rates increase progressively during normal pregnancy as part of normal placental development (13). In addition, it has been demonstrated that apoptosis increases in pregnancies complicated by some pathologies such as preeclampsia, fetal growth restriction and diabetes (1, 13, 19, 26). Although the exact mechanism and full complement of regulatory factors involving apoptotic cell death in the human trophoblast layer are unknown, many molecules are associated with the induction and prevention of apoptosis in different models (13, 27, 28). Bax is one such molecule, whose expression is considered a pro-apoptotic factor, responsible for evoking or increasing apoptosis. In particular, we previously demonstrated that Bax increased from the first to the third trimester of gestation in normal gestation (9). In accordance with this event, some authors (9, 29) have demonstrated that the expression of the anti-apoptotic factor, Bcl-2, diminishes as gestation progresses, suggesting that a parturition-associated, biological change might induce apoptosis in the placental villi (13). In the present paper, we first compared Bax expression among the first trimester voluntary termination of pregnancy and the first trimester abortion showing a strong increase of Bax expression in the cytotrophoblast, stroma, endothelial cells and decidua of placentas of the first trimester abortion compared to the low/moderate Bax immunopositivity in all the placental compartments during the first trimester voluntary termination of pregnancy. These data suggest that up-regulation of apoptotis may be involved in pregnancy complications until the abortion. Secondly, we compared the localization and the expression of Bax among third trimester spontaneous birth and caesarean birth demonstrating a more intense immunopositivity for Bax in the third trimester spontaneous birth respect to the third trimester caesarean birth. These results seem to suggest that apoptosis in placental villi may be a key factor in allowing normal delivery. Thirdly, we investigated the localization and the expression of Bax in pathological pregnancy conditions such as preeclampsia and diabetes. Specifically, in preeclampsia, we observed an increase of Bax expression in all the placental compartments compared to the normal full-term placentas. Preeclampsia affects 7-10% of all pregnancies and is a major cause of maternal and fetal morbidity and mortality (26, 30). In this disorder, the whole turnover of villous trophoblast is increased, commencing with increased proliferation of cytotrophoblast (1, 31). This alone could produce increased end stages of apoptosis in the syncytiotrophoblast (1, 6). According to this hypothesis, Han *et al.* (26) demonstrated

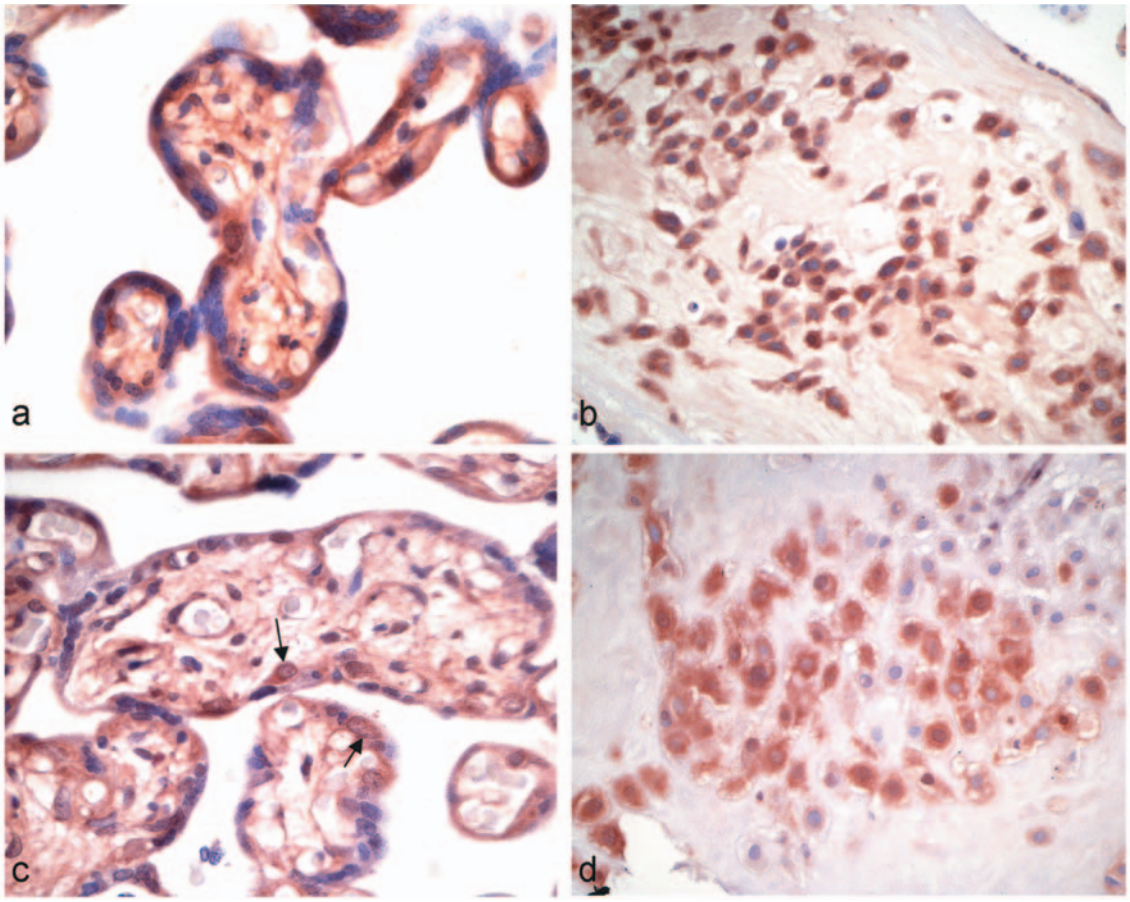

Figure 3. *Localization of Bax in human placenta of the third trimester of gestation. a) Bax immunopositivity in the placenta of third trimester spontaneous birth, x300; b) Intense immunopositivity in extravillous trophoblasts (EVTs) of the placenta of third trimester spontaneous birth, x150; c) Moderate Bax immunopositivity in cytotrophoblast (arrows) in the placental villous of the third trimester caesarean birth, x300; d) Moderate Bax immunopositivity in EVTs of the third trimester caesarean birth, x300.*

that caspase-10 and death receptor 3 (DR-3) were up-regulated in placenta from preeclamptic patients (26) suggesting that placental apoptosis and altered gene expression in the trophoblast may influence the pathogenesis of preeclampsia. Our results corroborate this finding. Regarding placenta from diabetic patients, we demonstrated a moderate Bax expression in all the placental compartments, slightly decreased compared to the normal full-term placentas. A relationship between hyperglycemia and apoptosis has been reported in a few studies (13). It has been demonstrated that there were no differences in cell death rates among the trophoblast compartments of diabetic and normoglycemic placentas (15). In contrast, Moley (32) showed that hyperglycemia up-regulates p53 and down-regulates the glucose transporters, GLUT1, 2 and 3, triggering the mitochondrial death cascade pathway. In addition, the oxidative stress induced by glucose deprivation triggers BAX-associated events, including subsequent caspase activation and progression of apoptotic cell death (5, 13).

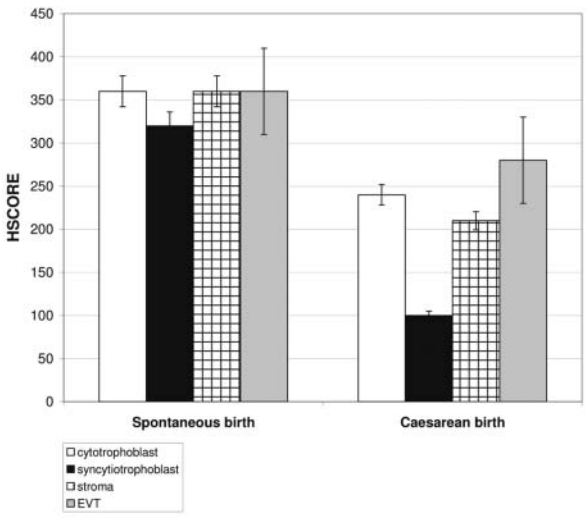

Figure 4. *Expression pattern of Bax immunopositivity in the third trimester spontaneous birth and in the third trimester caesarean birth. Vertical lines show S.E.M.*

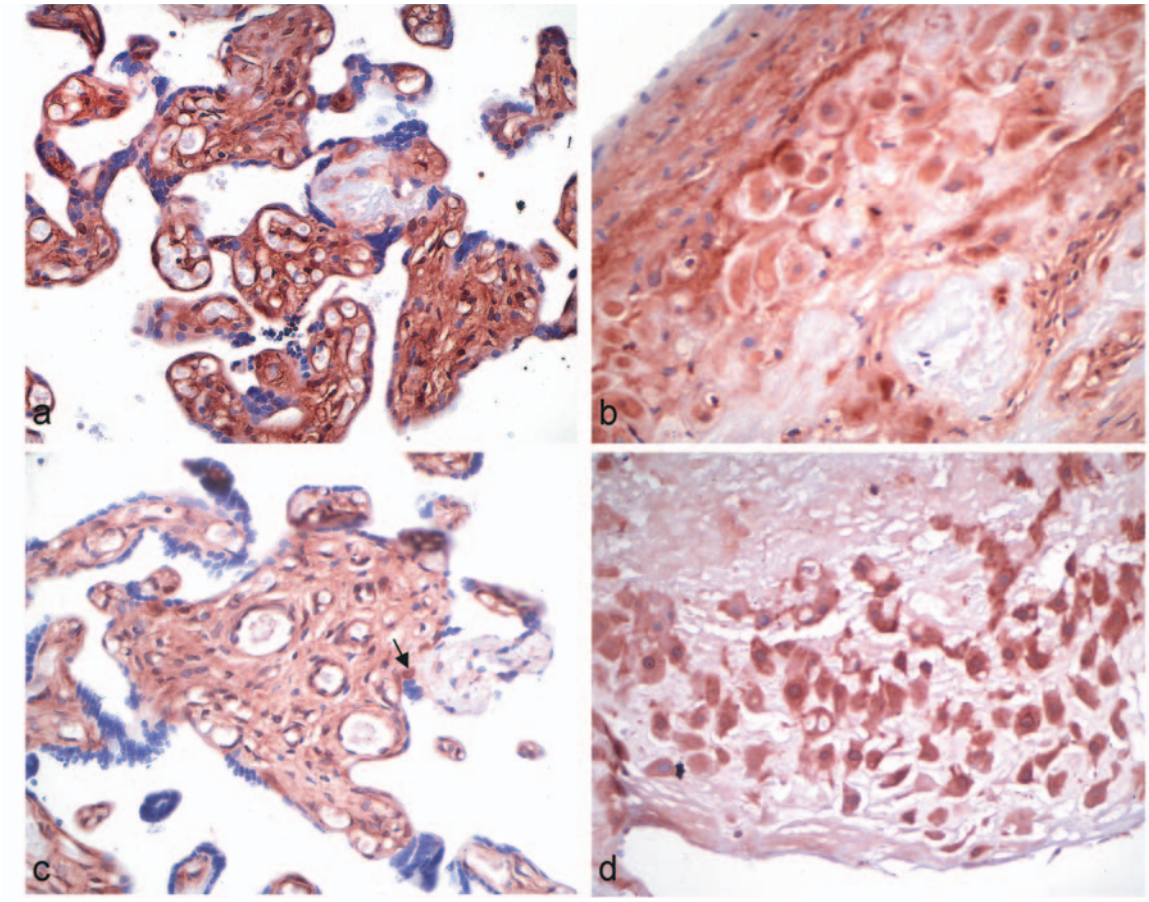

Figure 5. *Localization of Bax in human placentas of the third trimester of gestation, in preeclampsia and diabetes. a) Strong Bax immunopositivity in preeclamptic placenta of the third trimester, x150; b) Moderate Bax immunopositivity in EVTs of preeclamptic placenta of the third trimester, x150; c) Moderate Bax immunopositivity in cytotrophoblast (arrow) in the placental villous of diabetic placenta of the third trimester, x150; d) Intense Bax immunopositivity in EVTs of diabetic placenta of the third trimester, x150.*

Recently, Sgarbosa *et al.* (13) investigated the Bcl-2 expression in normoglycemic, diabetic and daily hyperglycemic full-term placentas. In particular, they demonstrated that apoptotic indices were inversely correlated with Bcl-2 expression (13). Specifically, the lower expression of Bcl-2 protein in term hyperglycemic placentas might result in the loss of protection against apoptosis, which may represent one of the altered mechanisms in placentas of diabetic patients (13). Taken together, these results seem to suggest that although apoptosis is a normal constituent of cell turnover in all trophoblast compartments, deregulation of this process may lead to placental dysfunction and pathologies.

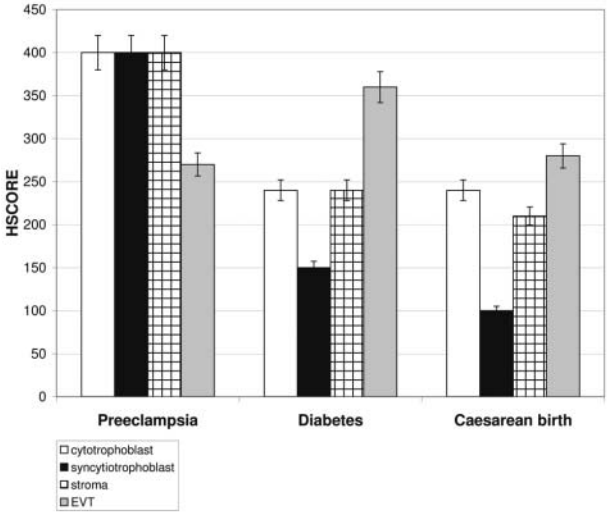

Figure 6. *Expression pattern of Bax immunopositivity in placentas in preeclampsia, diabetes and caesarean birth of the third trimester of gestation. Vertical lines show S.E.M.*

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