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Diabetes Research and Clinical Practice xxx (2006) xxx–xxx

DIABETES RESEARCH  
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CLINICAL PRACTICE[www.elsevier.com/locate/diabres](http://www.elsevier.com/locate/diabres)

## Changes in apoptosis and Bcl-2 expression in human hyperglycemic, term placental trophoblast

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Received 30 June 2005; received in revised form 5 December 2005; accepted 22 December 2005

### Abstract

Apoptosis and its associated regulatory mechanisms are physiological events crucial to the maintenance of placental homeostasis; imbalance of these processes, however, such as occurs under various pathological conditions, may compromise placenta function and, consequently, pregnancy success. Increased apoptosis occurs in the placentas of pregnant women with several developmental disabilities, while increased Bcl-2 expression is generally associated with pregnancy-associated tumors. Herein, we tested the hypothesis that apoptosis-associated disturbs might be involved in the placental physiopathology subjected to different maternal hyperglycemic conditions.

Thus, in the present study we investigated and compared the incidence of apoptosis using TUNEL reaction and Bcl-2 expression, in term-placentas of normoglycemic, diabetic and daily hyperglycemic patients. Tissue samples were collected from 37 placentas, being 15 from healthy mothers with normally delivered healthy babies, and 22 from mothers with glucose disturbances. From these latter 22 patients, 10 showed maternal daily hyperglycemia and 12 were clinically diabetics. Both Bcl-2 expression and apoptotic DNA fragmentation were established and quantified in the trophoblasts of healthy mothers. Compared to these reference values, a higher apoptosis index and lower Bcl-2 expression were disclosed in the placentas of the diabetic women, while in the daily hyperglycemic group, values were intermediate between the diabetic and normoglycemic patients. The TUNEL/Bcl-2 index ratio in the placentas varied from 0.02 to 0.09 for pregnant normoglycemic and diabetic women, respectively, revealing a predominance of apoptosis in the diabetic group. Our findings suggest that hyperglycemia may be a key factor evoking apoptosis in the placental trophoblast, and therefore, is relevant to diabetic placenta function.

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**Keywords:** Diabetes; Cell death; Proliferation; Syncytiotrophoblast; TUNEL reaction

### 1. Introduction

Programmed cell death by apoptosis and its associated regulatory mechanisms are intimately involved in placental homeostasis, growth and remodeling [1,2]. The analyses of these processes expand our knowledge of placental physiology.

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The Bcl-2 protein family is one of the main groups of molecules that play a significant role in the regulation of apoptosis. Some proteins from this family, including Bcl-2, inhibit programmed cell death, while others, like Bax and Bak, promote it. Bcl-2 proteins seem to modulate cellular sensitivity to apoptosis since they complex with pro-apoptotic proteins [3–5].

Apoptosis and proteins associated with the regulatory pathway of cell death have been studied in the villous and extravillous trophoblasts of the human placenta, in both normal and complicated pregnancies [1,6–23]. However, the dynamics of apoptosis events in the placental development under different conditions is still controversial. According to Halperin et al. [10], Straszewski-Chavez et al. [21] and Smith et al. [1] the apoptotic rate increases progressively during normal gestation, being interpreted as part of normal placental development. In contrast, Ishihara et al. [13] and Sakuragi et al. [19] found that placental apoptosis diminishes after week 5 of gestation and correlated these data to the prevalence of Bcl-2 expression mainly in the syncytiotrophoblast layer. Adding to that, Yamada et al. [23] showed that diminished apoptosis in the normal human placenta is dependent on the maternal age as an alternative to compensate the lower functions of the placental cells in aged-mothers.

An abnormal level of apoptosis also has been correlated with a great variety of gestational pathologies such as in the placentas of abortions, ectopic pregnancy, intrauterine growth retardation, post-term pregnancy, preeclampsia and maternal hypertension syndrome [10,15,16,20]. Hyperglycemia modulates the expression of apoptosis regulatory genes in the preimplantation blastocyst stage [24] and is able to inhibit the proliferation and to change the mitochondrial activity in trophoblast cell lines [25]. Different time points in the development, however, does not seem to follow the same hyperglycemia-associated mechanisms. Reactive oxygen species produced as a result of exposure to hyperglycemia in diabetes, decrease the apoptotic index of the villous placental tissues in comparison with normal pregnancies [6]. Investigations with animal models have also shown that in streptozotocin-induced diabetic rats, placental growth is markedly altered, exhibiting a notable decrease in apoptotic and proliferation indices [26].

Considering that glycemic disturbance is associated with abnormal fetal and placental development [6,24,26] and that apoptosis/Bcl-2 expression balance are relevant factors involved in the regulation of placental growth [17,18], we hypothesized that apop-

toxis-associated disturbs might be involved in the placental physiopathology under different maternal hyperglycemic conditions. Thus, in the present study we describe differences in apoptotic rates and the expression of Bcl-2 in full-term placentas in normoglycemic, diabetic and daily hyperglycemic pregnant women.

## 2. Methods

### 2.1. Subjects

The Ethics Committee for Research of the Botucatu Faculty of Medicine – UNESP (Brazil) approved all procedures; the pregnant women also formally consented to sample collection and the study goals. Samples of term placenta villous tissue were obtained from the Department of Obstetrics, São Paulo State University (UNESP). Placental tissues were taken from 37 pregnant women allocated in the following groups: maternal daily hyperglycemic ( $n = 10$ ), diabetic ( $n = 12$ ) and normoglycemic, pregnant women ( $n = 15$ ). Samples were obtained from term pregnancies at 36–40 weeks of gestation (mean  $38.42 \pm 0.38$  weeks), immediately after labor and vaginal delivery. The maternal age ranged from 20 to 32 years of age (mean  $27.92 \pm 2.54$  years). Gestational diabetic pregnant women were defined using the glucose tolerance test (100 g OGTT) [27], and maternal daily hyperglycemic patients by normal OGTT and altered glucose profile [26]. The glucose titers in these patients were higher than 90 mg/dL for fasting and/or higher than 130 mg/dL for post-prandial tests. The diagnostic tests were performed between weeks 26 and 28 of pregnancy in all positive, screened patients [27]. The normoglycemic group included uncomplicated term pregnancies; healthy mothers normally delivered healthy babies with normal OGTT and normal glucose profile. Diabetic and daily hyperglycemic patients were treated through diet alone, or if necessary, by diet associated with insulin to establish normoglycemia.

### 2.2. Tissue collection and processing

Immediately after vaginal delivery, fragments consisting of villi were taken from the central placental cotyledon, near the umbilical cord, free of macroscopic changes and fixed in 10%-buffered formalin for 24 h. Samples were routinely processed for paraffin embedding. Five micrometre-thick sections were deparaffinized and mounted on poly-L-lysine-coated slides and processed for the following analyses.

#### 2.2.1. TUNEL assay

DNA strand breaks were detected by TUNEL assay using a commercial kit (Apoptag Plus In situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD, USA). Briefly, de-waxed and rehydrated placental sections were pretreated with proteinase K (10  $\mu\text{g}/\text{mL}$ ) in 0.05 M Tris–HCl buffer, pH 7.4, for 30 min at 37 °C, washed in phosphate-buffered saline (PBS, Sigma

Chemical Co, St. Louis, MO, USA) and incubated in a reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and dUTP (fluorescein-labeled deoxyuridinetriphosphate) in a humidity chamber for 1 h, at 37 °C. Incorporated fluorescein was detected using an anti-fluorescein-antibody conjugated with peroxidase, which was developed with diaminobenzidine–hydrogen peroxide (Sigma Chemical Co, St. Louis, MO, USA). TUNEL-positive cells were identified by their brown nuclear staining. The sections were counterstained with 50% Harris' hematoxylin solution, and mounted in Entellan medium. TdT was omitted from the labeling reaction in the negative controls.

### 2.2.2. Immunohistochemical localization of Bcl-2 protein

Dewaxed and rehydrated placental sections were immunostained for Bcl-2, using an avidin–biotin immunoperoxidase method. Briefly, the sections were sequentially treated with 3% hydrogen peroxide in distilled water for 10 min at room temperature to inactivate endogenous peroxidase activity, 1% BSA–PBS for 1 h at 37 °C to quench non-specific reactions, mouse anti-human Bcl-2 monoclonal antibody (Oncogene, Boston, MA, USA; diluted 1:20 in PBS) for 18 h at 4 °C, biotinylated rabbit anti-mouse (Vector, Burlingame, CA, USA; diluted 1:200 in PBS) for 1 h at 25 °C, and avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Vector, Burlingame, CA, USA, diluted 1:50 in PBS) for 45 min at 25 °C. Peroxidase activity was detected by color development with diaminobenzidine–hydrogen peroxide as the chromogen (Sigma Chemical Co., St. Louis, MO, USA). The sections were lightly counterstained in hematoxylin. Control reactions were prepared by omitting the primary antibody.

### 2.2.3. Quantitative analysis

Sections were examined using a light microscope with a 100× objective lens. Five randomly selected fields from each tissue section were digitalized at 1000× final magnification. A total of 10 sections from each group (normal, diabetic and hyperglycemic) were blinded analyzed.

All TUNEL-stained nuclei in the villous trophoblast (mainly syncytiotrophoblast, since the villi of full term placentas show very few cytotrophoblast cells) were counted in each field randomly selected. The TUNEL index was expressed as the percentage of positively staining nuclei in the total number of trophoblast nuclei per field.

Because one of the most significant morphological characteristics of syncytiotrophoblast is multiple nuclei sharing the same cytoplasm and Bcl-2 is diffusely expressed in the cytoplasm, to maintain the same pattern of measuring units as TUNEL reaction, the Bcl-2 expression index was calculated as the percentage of villous trophoblast nuclei per field of tissue section that exhibited intense, brown perinuclear immunoperoxidase staining, regardless whether the nuclei were from the cytotrophoblast cells or from syncytiotrophoblast.

The ratio between the TUNEL and Bcl-2 expression indices was also calculated as an additional parameter to further explore the balance between apoptosis-anti-apoptosis/Bcl-2 expres-

sions associated with maternal hyperglycemic alterations. The TUNEL:Bcl-2 ratio for the normoglycemic group was set at 1, and was used to estimate the ratio for the other groups.

### 2.2.4. Statistical analysis

The TUNEL and Bcl-2 expression indices were expressed as mean ± S.E.M. The data were compared using the InStat Statistical Software Package (GraphPad Software Inc., CA, USA) and non-parametric analysis (Kruskal–Wallis test). The minimum of significance level was set at  $P = 0.05$ .

## 3. Results

### 3.1. TUNEL positive trophoblast nuclei

Apoptosis was assessed using the in situ DNA 3'-end labeling assay and was apparent in the nuclei of the villous trophoblast in all three groups. Labeling in the syncytiotrophoblast was also found in the syncytial knots (Fig. 1).

The apoptotic indices in the different groups are shown in Table 1. The incidence of TUNEL-positive nuclei tended to be higher in the diabetic and daily hyperglycemic groups compared to the normoglycemic, pregnant women. The statistical analysis, however, revealed significant differences only between the normoglycemic and diabetic groups ( $P \leq 0.01$ ).

### 3.2. Trophoblast Bcl-2 expression

Bcl-2 was immunoenzymatically detected in all groups studied as a strong, brown, cytoplasmic stain (Fig. 1). Frequently, the trophoblast lining the villi exhibited positive cytoplasmic areas, continuous with non-labeled cytoplasm, surrounding adjacent nuclei in the syncytiotrophoblast. The cytoplasm of the syncytial knots also occasionally showed Bcl-2 expression.

To establish the rates of Bcl-2 expression in the trophoblast, the number of nuclei surrounded by the brownish immunoreaction shown in Fig. 1 were quantified. A decrease in the mean number of immunopositive cells was found in the placental villi of the hyperglycemic and diabetic women, compared to the normoglycemic group (Table 1). Significant differences were only found between the normal and diabetic groups ( $P \leq 0.01$ ).

### 3.3. TUNEL-positive nuclei:Bcl-2 expression index ratios

The TUNEL/Bcl-2 index ratios were 0.02, 0.05 and 0.09, respectively, for the placental villi of

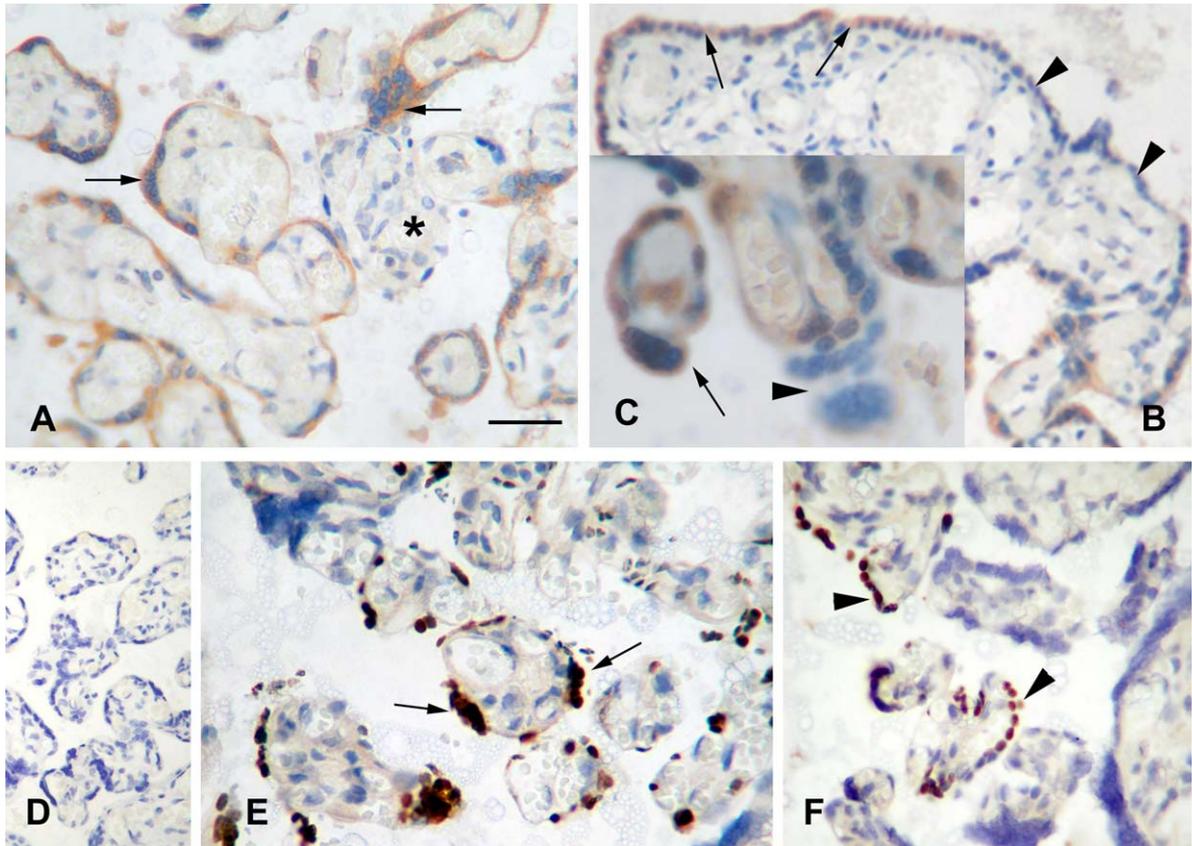


Fig. 1. Full-term human placental villi. (A–D) Immunohistochemical localization of Bcl-2 protein. (A) Note the Bcl-2 positive brownish coloration (arrows) in the syncytiotrophoblast cytoplasm. Juxtaposed stained and unstained villi (A, \*) can be seen, as can (B) stained (arrows) and unstained (arrowheads) areas in the same villus. Cytoplasmic labeling also varies in the syncytial knots (C, arrow and arrowhead). (D) In the negative control, from which the primary antibody was omitted, no immunolabeling is observed. (E and F) TUNEL reaction. TUNEL-positive nuclei are seen in the syncytiotrophoblast layer (arrowheads) and in the syncytial knots (arrows). The micrographs are representative of placental samples from diabetic (A and B), daily hyperglycemic (B and F) and normoglycemic (C and D) pregnant women, respectively. The scale bar in figure (A) represents in (A and B), (E and F), 40  $\mu\text{m}$ ; 20  $\mu\text{m}$  in (C) and 80  $\mu\text{m}$  in (D).

Table 1

Incidence of TUNEL-positive nuclei and Bcl-2 expression in the villous trophoblasts of normoglycemic, daily hyperglycemic and diabetic, pregnant women

Group	Apoptosis index (%)	Bcl-2 index (%)
Normoglycemic ( $n = 24$ )	$1.98 \pm 0.71$	$95.76 \pm 1.86$
Daily hyperglycemic ( $n = 11$ )	$4.62 \pm 1.12$	$81.04 \pm 6.5$
Diabetic ( $n = 20$ )	$6.18 \pm 1.50^*$	$71.44 \pm 8.3^*$

Data are the mean + S.E.M. Apoptosis index (%): number of TUNEL positive trophoblast nuclei per 100 nuclei; Bcl-2 index (%): number of trophoblast nuclei that exhibited intense, brown perinuclear immunoperoxidase staining per 100 nuclei.

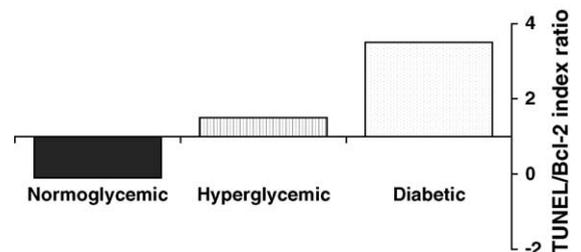


Fig. 2. Bcl-2 expression and TUNEL-positive cell index ratios. The values for the normoglycemic index were normalized to 1.0 and those for daily hyperglycemia and diabetes calculated proportionally. The values are presented as arbitrary units.

normoglycemic, daily hyperglycemic and diabetic pregnant women. The standardized values are plotted in Fig. 2.

#### 4. Discussion

Apoptosis has been demonstrated in the human placenta under normal physiological and pathological conditions [1,2,6,7,10,12,13,15–23,25,28]. Several studies attempted to establish a correlation with the apoptotic indices in these states. Here, we demonstrate the balance between apoptosis and expression of the anti-apoptotic protein Bcl-2 in the villous area of full-term placentas, in normoglycemic, diabetic and daily hyperglycemic patients, in an attempt to further understand the changes in placental development in women exhibiting different hyperglycemic disturbances.

The rates of placental apoptosis even in normal human gestations are still controversial. While various studies have shown a significant increase in apoptosis as pregnancy progresses [1,10,21], others have demonstrated that programmed cell death predominates during early pregnancy, drastically diminishing after the second trimester [13,19]. Employing the TUNEL method, we established a mean rate of 1.98% for the villous area in term placentas that is slightly higher than the rates reported by Chan et al. [2], Burleigh et al. [6] and Yamada et al. [23]. Owing to the temporal characteristics of the term placenta, in this study, the evaluation of the apoptotic indices mainly favored the syncytiotrophoblast in the villous trophoblast layer, which does not exclude cytotrophoblast cells, when present. Apoptotic stromal and endothelial cells were systematically excluded.

The current study also showed an elevation in the incidence of apoptosis in the placentas of hyperglycemic women, although, statistically increased only compared to diabetic women. A relationship between hyperglycemia and apoptosis has been reported in a few studies. Moley [24] showed that hyperglycemia upregulates p53 and downregulates 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 [12]. Indeed, cultured trophoblast cell lines respond to hyperglycemic conditions increasing apoptosis rate [25]. Controversially, data from the literature also support opposite results. Burleigh et al. [6] did not find differences in cell death rates among the trophoblast compartments of five diabetic and normoglycemic

placentas. Although apoptotic cells were determined by the same method, perhaps differences in maternal age and tools and techniques to maintain normoglycemia may explain the discrepancies in our results. In normal gestations a dramatic fluctuation in the apoptotic index and Bcl-2 expression is seen throughout the course of gestation, ranging from 1.8 to 34.6% [19]. Furthermore, experimental evidence has also indicated that insulin treatment for hyperglycemic mice can prevent the embryotoxic effects of glucose *in vivo*, which include apoptotic cellular changes [28]. In our study, diabetic and daily hyperglycemic pregnant women were treated through diet alone and only occasionally, when necessary, through diet associated with insulin to establish normoglycemia. Therefore, we cannot exclude the possibility that the absence or eventual administration of insulin has resulted in higher apoptotic rates in comparison to other systematically treated, diabetic patients.

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 [29,30]. Bcl-2 is one such molecule, whose expression is considered an anti-apoptotic factor, responsible for preventing or minimizing apoptosis [17–19].

In normal placentas, expression of the Bcl-2 protein is detectable throughout the villous syncytiotrophoblast in contrast to the expression of BAX, a pro-apoptotic protein [18]. Developmental changes in Bcl-2 expression also occur. Kim et al. [14] reported that Bcl-2 expression diminishes as gestation progresses, and suggested that a parturition-associated, biological change might induce apoptosis in the placental villi. An inverse relationship also has been identified between apoptosis and Bcl-2 expression in the syncytiotrophoblast [22]. Our results corroborate these findings; apoptotic indices were inversely correlated with Bcl-2 expression in all experimental groups. However, in maternal hyperglycemia, the lower of Bcl-2 expression, revealing the predominance of apoptosis over non-apoptotic processes, accompanied higher incidences of placental apoptosis. Ishihara et al. [13] suggested that the abundant expression of Bcl-2 protein in syncytiotrophoblast in term placentas may be one of the regulatory processes of apoptosis and this may be indispensable for the maintenance of pregnancy. In this context, the lower expression of Bcl-2 protein in term hyperglycemic placentas might result in the lost of protection against apoptosis, which may represent one of the altered mechanisms of diabetic placentas.

In conclusion, although further studies are necessary to elucidate the precise mechanisms involved in alterations of placental functions in maternal hyperglycemia, our findings suggest that this condition may be a key factor in evoking apoptosis and, therefore, is relevant to normal placental growth, gestational success, and the prevention of adverse perinatal outcomes. Further, the TUNEL/Bcl-2 ratio may provide an additional parameter for early diagnosis and adequate therapeutic treatment associated with fetal weight deviations affected by changes in the maternal–placental–fetal interface.

## Acknowledgment

CAPES supported this study.

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