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Effect of IFNγ on caspase-3, Bcl-2 and Bax expression, and apoptosis in rabbit placenta☆

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Abstract

The purpose of this study was to determine whether apoptosis in placenta was affected by IFNγ, which can induce abortion, and whether the effect of IFNγ on apoptosis resulted from an intrinsic program of apoptosis, which was regulated by Bcl-2 and Bax. DNA fragmentation analysis indicated that cleavage of DNA into 180 bp and its polymers were recognized in placenta in control and IFNγ treated groups. Quantitative analysis of low molecular weight fragments of DNA revealed a significant increase in cases of 100,000 IU IFNγ treatment compared with those in normal pregnancy ($P<0.05$). An analysis in situ revealed that apoptosis occurred predominantly in syncytiotrophoblast. Expression of Bcl-2 and Bax in placenta was evaluated by immunoblot analysis and immunohistochemistry study. Bcl-2 was expressed predominantly in syncytiotrophoblast, and was not expressed in cytotrophoblast of all cases. Whereas Bax was expressed in cytotrophoblast, syncytiotrophoblasts were found to be negative for Bax protein expression in all cases. Both Bcl-2 and Bax expression was decreased 0.44 fold and 0.46 fold by 50,000 IU IFNγ and 0.41 fold and 0.03 fold by 100,000 IU IFNγ. This resulted in change of a 0.07 fold increase in the Bax : Bcl-2 ratio in 50,000 IU IFNγ treated groups and 0.41 fold increase in 100,000 IU IFNγ treated groups as compared with those in control groups. The difference in Bax to Bcl-2 ratio between control and 100,000 IU IFNγ treated groups was significant ($P<0.05$). The localization of caspase-3, the executioner of apoptosis, was detected in some cytotrophoblast and syncytiotrophoblast and increased 0.03 fold and 0.68 fold in 50,000 IU IFNγ and 100,000 IU IFNγ treated groups, respectively. There was significant difference between control and 100,000 IU IFNγ treated groups ($P<0.05$). The results showed that high dose of IFNγ administration increased the extent of apoptosis in placenta, the Bax to Bcl-2 ratio, and the activated caspase-3.

Keywords: IFNγ; Placenta; Apoptosis; Bcl-2; Bax

1. Introduction

Apoptosis is an intrinsic cellular mechanism leading to self-destruction of a cell [1,2]. It is a normal physiological phenomenon that is required in embryogenesis and cellular stresses to remove injured cells and to counter abnormal cell proliferation in different pathological situations [2,3]. The mechanisms responsible for regulating apoptosis involve a precisely controlled series of steps that have been remarkably conserved through evolution and that become activated in the cell poised to die [4–6]. Expression of proto-oncogene, Bcl-2, and its related protein, as well as Bax, plays important roles in those mechanisms.

Bcl-2 functions to prevent cell death, whereas Bax, which forms hetero-dimers with Bcl-2, appears to accelerate the cell death signal [7]. The ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax determines the susceptibility of a given cell to apoptosis [5]. However,

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Bcl-2 and Bax may also function independently to control cell death [8,9]. Multiple Bcl-2 related proteins have been identified in humans, highlighting the complexity of apoptosis regulation [10].

Cell execution of apoptosis process is accomplished by activation of caspases, a family of cysteine proteases which are regulated by Bcl-2 family members and which serve as the final effectors in apoptosis [4–6,11]. The enzymes are an evolutionarily conserved, constitutively expressed group of proteases with specificity for aspartic acid residues in their substrate [12]. Phylogenetic analysis has revealed that caspases may be grouped into two major subfamilies: the ICE and CED-3 subfamilies. Members of the ICE subfamily (caspases 1, 4, 5, 11–13) may have subsidiary roles in pro-inflammatory events such as cytokine mobilization, whereas members of the CED-3 subfamily (caspases 2, 3, 6–10 and 14) are primarily involved in apoptosis [13]. Huppertz et al. [14] reported that caspases 3, 6–8 and 10 are expressed in placenta. Caspase-3, considered as one of the central executioner molecules, is responsible for cleaving various proteins thereby disabling important cellular structural, functional and repair processes.

Placenta regulates maternal–fetal gas, nutrient and waste product exchange. In the recent reports, apoptosis is identified in the trophoblast of human placenta villi and apoptotic mechanisms play a central role in placenta turnover [15,16]. A higher-than-normal incidence of apoptosis was detected in placentae of pregnancies complicated by intrauterine growth restriction and/or spontaneous abortion [17]. Dysregulation of apoptosis in this key cellular interface might be a primary pathological event.

Interferon gamma (IFNγ), a product of activated T lymphocytes and natural killer (NK) cells, is best known for its immunological functions. It has been widely applied to cure many viral diseases including hepatitis [18], human immunodeficiency [19] and cervical intraepithelial neoplasia [20]. However, the application of IFN-γ to pregnant female would exert its deleterious effect on pregnancy. Several papers suggested that administration of IFN-γ resulted in miscarriage in rat [21], increased abortion rate as high as 75% in cross pairings of two strains of mice (CBA×DBA) [22] and made a drop of 80% embryo implantation rate in rabbit [23]. The role of IFNγ in pregnancy has not been studied in detail, but some papers suggest that IFNγ can enhance the effect of TNFα by inducing apoptotic cell death in cultured trophoblast cells [24] and IFNγ can induce apoptosis in differentiated human leukemic B cell lines [25]. Taken together, we speculated that IFNγ might increase apoptotic cell death in placenta and the effect might result from the increase of Bax to Bcl-2 ratio. To test this hypothesis, we studied the influence of IFNγ on the extent of apoptosis in placenta and the expression of Bcl-2, Bax and active caspase-3.

2. Materials and methods

2.1. Animals

Nine sexually mature healthy female rabbits (Newsland) were subjected to this study. Individual estrous female rabbits were caged overnight with a Newsland male rabbit. The next day was designated as day 1 (g.d. 1) of pregnancy.

2.2. Treatment groups

Pregnant rabbits received vaginal edge muscular injections of 50,000 or 100,000 IU recombinant IFNγ (Boeringer Mannheim, Mannheim, Germany) at g.d. 6 (just before implantation). These doses were chosen to replicate the protocols of a previous study [23]. The control groups received an equal volume (100 ul) of saline. At g.d. 12, the animals were killed. Some placentae ($n = 9$) were frozen in eppendorf tubes and stored at −80 °C until processing for DNA extraction and immunoblot analysis. Some placentae ($n = 9$) were fixed in 4% buffered parafomaldehyde for TUNEL tests and immunohistochemical study.

2.3. Isolation of nuclear DNA for apoptotic fragmentation analysis

Total DNA was extracted by slightly modifying a previously described protocol [26]. Placenta ($n = 3$ from each group) DNA was isolated first in 10 mM Tris–Cl, 100 mM EDTA, 0.5% SDS, pH 7.5, as the extraction buffer, followed by extraction in phenol, chloroform and a final ethanol precipitation. After RNase A (QIAGEN Inc., Valencia, CA, USA) digestion at 100 μg/ml for 1 h at 37 °C, identical amounts of DNA were separated on 2.0% agarose gel electrophoresis. Then the gel was stained with ethidium bromide (1 μg/ml) and was photographed. The photographs were scanned and the intensity of band fluorescence was measured using Fragment NT Analysis Micro software (Molecular Dynamics, Sunnyvale, CA, USA). Nucleosomal DNA fragmentation values were expressed as the ratio of the intensity of the low molecular weight DNA fragmentation (≤15 kb) to the high molecular weight DNA (>15 kb).

2.4. TUNEL test of histologic sections using confocal analysis

Placentae ($n = 3$ from each group) in 4% buffered paraformaldehyde solution for 5 h were removed and placed in 15% sucrose solution overnight. After fixation, 10 μm sections were cut on a freezing microtome. The sections were subjected to TUNEL test, using In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany). Briefly, cryopressured

tissue sections were permeabilized (0.1% Triton X 100 in 0.1% sodium citrate) for 5 min. Deoxynucleotidyl transferase and fluorescein-conjugated dUTP in TdT buffer were added to cover the sections which were then incubated in a humid chamber at 37 °C for 1 h. After washing, the sections were counterstained with propidium iodide (PI) (Sigma Chemical Company, St Louis, MO, USA). Positive control samples were prepared by incubating sections with DNase I (Sigma Chemical Company, St Louis, MO, USA) prior to treatment with terminal transferase. Negative controls were specimens to which deoxynucleotidyl transferase had not been added. Then the sections were analyzed for apoptotic DNA fragmentation using confocal analysis.

2.5. Immunohistochemistry

Immunohistochemical studies were performed on 4% buffered paraformalin-fixed, 10 μm thick cryosections. Three sections from each placenta were subjected to immunohistochemical study. Endogenous peroxidase activity was blocked by a 5 min treatment with 3% hydrogen peroxide in absolute methanol. Sections were then incubated in a humid chamber with the primary antibodies for 1 h at room temperature. The following primary mouse monoclonal antibodies were used in each case: Bcl-2 (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA), actin (2 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and caspase-3 (4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA). To minimize background staining, sections were preincubated with a 1 : 10 dilution of normal goat serum. To enhance the immunostainning, sections were digested with 10 μg/ml protease K (Sigma Chemical Company, St Louis, MO, USA) in phosphate-buffered saline, pH 7.6, for 15 min before incubating with the primary antibody. Sections were then counterstained with 0.5% hematoxyline (Sigma Chemical Company, St Louis, MO, USA), then destained briefly in HCl–alcohol before dehydration and mounting under glass coverslips with permount. To evaluate the specificity of the antibodies, negative control staining was performed by substituting mouse Ig-G with the primary antibodies.

2.6. Western blotting analysis

Placenta tissue samples ($n = 3$ from each group) were homogenized after addition of 500 ul sterilized water. The samples were then centrifuged at 12,000 rpm/min for 10 min at 4 °C. For the procedure of western blot analysis see the reports of Dai et al. [27].

2.7. Protein dot blotting

Placenta tissue samples ($n = 3$ from each group) were homogenized after the addition of 500 ul sterilized water, and were suspended on ice for 30 min. The samples were then centrifuged at 12,000 rpm/min for 15 min at 4 °C. The protein content in supernatants was determined by the BioRad protein assay (BioRad, Hercules, CA, USA). The following protein dot blot was carried out according to the procedures described by previous reports [28,29]. Aliquots of equalized amounts of protein were loaded in the wells of the Bio-Dot microfiltration units and allowed to filter through the nitro-cellulose membrane by vacuum. The membrane was removed from the apparatus and was then blocked in TBS (500 mM NaCl, 15 mM Tris–Cl, pH 7.5) containing 3% gelatin for 2 h. The blocking solution was removed and replaced with fresh solution containing 1% gelatin and reacted with 0.5 μg/ml monoclone antibody anti Bcl-2, Bax, β-actin and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation for 3 h at 37 °C, the membrane was washed three times in TBS and incubated for 2 h with peroxidase-conjugated protein A (1 : 1000) in TBS containing 1% gelatin. After three washes in TBS, membrane was developed by using the ECL western blotting detection system (Amersham International, Amersham, UK), according to the manufacturer's instructions. The membrane was then scanned and qualified by densitometry using Fragment NT Analysis Micro software (Molecular Dynamics, Sunnyvale, CA, USA).

2.8. Statistical analysis

Values are reported as the mean ± SEM. Statistical analysis was made by one-way ANOVA, and when significant treatment effects were indicated, the Student–Newman–Keuls multirange test was employed to make pairwise comparisons of individual means.

3. Results

3.1. Apoptosis induced by IFNγ in placenta

DNA ladders, characteristic of the apoptotic cleavage of nuclear DNA into small fragments between nucleosomes, were generated in placentae of all groups. Quantitative analysis of the small DNA fragments (≤15 kb) did not reveal significant change between normal and 50,000 IU IFNγ treated pregnant rabbits. By contrast, a significant increase ($P < 0.05$) was demonstrated in 100,000 IU IFNγ treated rabbits compared with those in normal pregnant rabbits (Fig. 1, A1).

To localize the apoptotic cells in placenta, selected sections from each group were subjected to confocal analysis. Analysis of the staining for placenta in treatment and control groups confirmed that apoptosis occurred in the syncytiotrophoblast, while cytotrophoblast was rarely detected for apoptosis (Fig. 1, A2–A4).

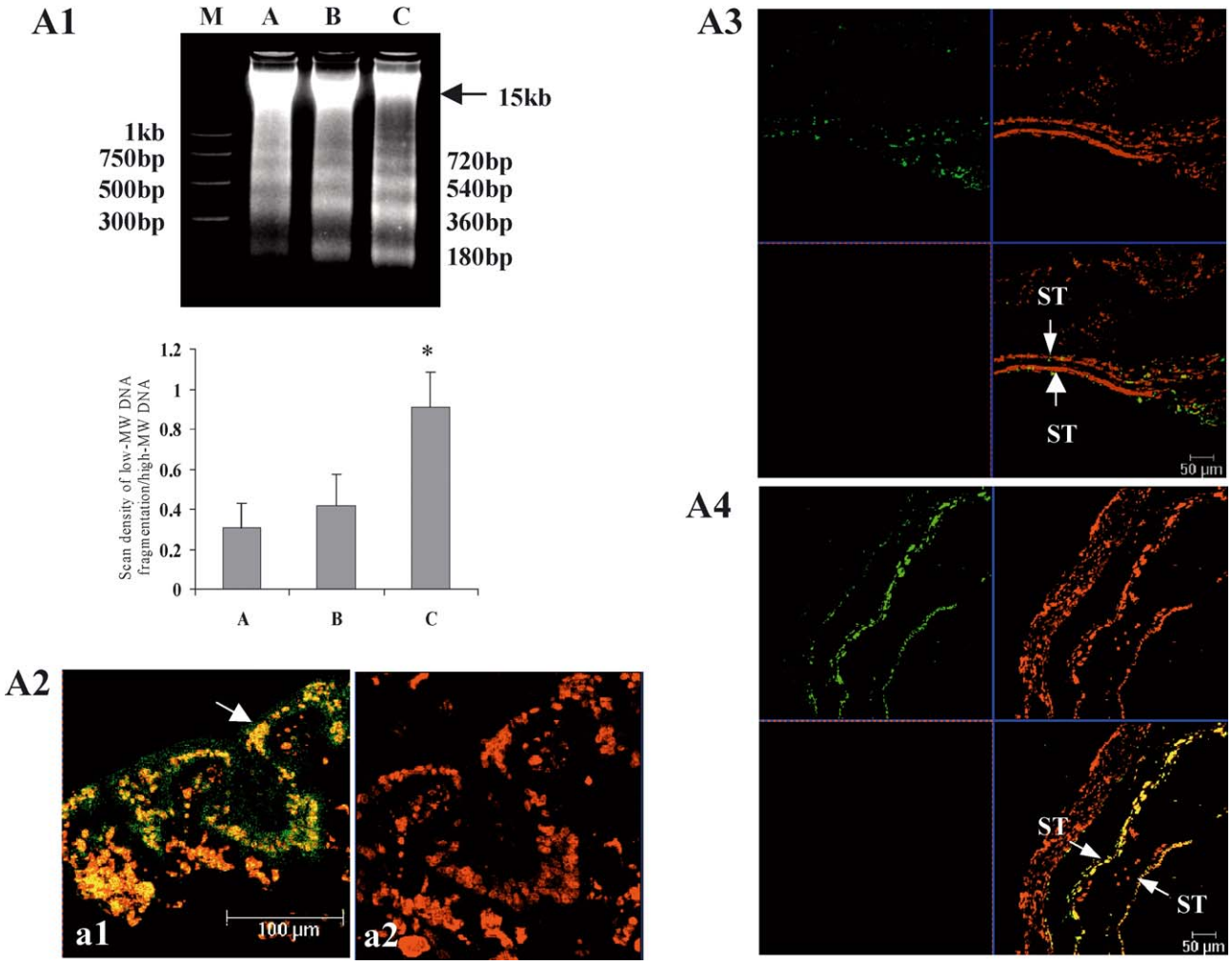

Fig. 1. Apoptosis in rabbit placenta. A1. Apoptotic fragmentation of DNA in placenta. Placenta tissues were obtained from g.d. 12 pregnant rabbit. DNA was isolated from placenta tissues and qualified by densitometry using Fragment NT scan software. An arrow indicates the point at which gels were cut for quantitation of the high and low-MW DNA. The DNA fragmentation values are expressed as the ratio of the intensity of the low-MW DNA to the intensity of the high-MW DNA. Statistical analysis was performed using ANOVA followed by the Student–Newman–Keuls multirange test. *Means significant change compared with control groups. (A) DNA from normal placenta (three cases); (B) DNA from placenta in rabbit treated with 50,000 IU IFNγ on g.d. 6 (just before implantation) and killed on g.d. 12 (three cases); (C) DNA from placenta in rabbit treated with 100,000 IU IFNγ on g.d. 6 and killed on g.d. 12 (three cases). A2. In situ 3′-end-labelling of DNA in the sections of placenta. The placenta tissues were obtained from g.d. 12 pregnant rabbit. Each sample was fixed in 4% buffered paraformaldehyde, and in situ 3′-end-labelling of DNA with fluorescein-conjugated dUTP was performed. Incorporation of fluorescein-conjugated dUTP into 3′-ends of DNA was detected with confocal analysis. The sections were counterstained with PI, as described in Section 2. The incorporation of fluorescein-conjugated dUTP is represented by the green coloration and such staining was the result of the apoptotic cleavage of DNA. The red fluorescence represented the nucleus of all cells in tissue. (a1) In normal and IFNγ treated placenta (nine cases) apoptosis localized predominantly in syncytiotrophoblast in all cases (arrow) and little apoptosis was detected in cytotrophoblast; (a2) in negative control section of placenta, deoxynucleotidyl transferase was not added. A3–A4. The effect of IFNγ on apoptosis in placenta during early gestation using TUNEL. A3, control; A4, treated with 100,000 IU IFNγ. ST, syncytiotrophoblast.

3.2. Localization of Bcl-2 and Bax protein and the enhancement of Bax to Bcl-2 ratio by IFNγ

Bcl-2 protein was localized predominantly to the syncytiotrophoblast cytoplasm in placenta in both normal and IFNγ treated groups. Bcl-2 staining was generally undetectable or expressed at low levels in cytotrophoblast. However, Bax protein was localized to limited cytotrophoblast. Bax was not expressed in the syncytiotrophoblast and in most of the cytotrophoblast (Fig. 2).

The results of Western blotting analysis showed that there was a special band respectively in responded location of molecular weight. The result showed specificity of antibodies of Bax and Bcl-2 (Fig. 3). Immunoblot analysis showed that either Bcl-2 or Bax expression was decreased 0.44 fold and 0.46 fold, respectively, by 50,000 IU IFNγ administration and 0.41 fold and 0.03 fold,

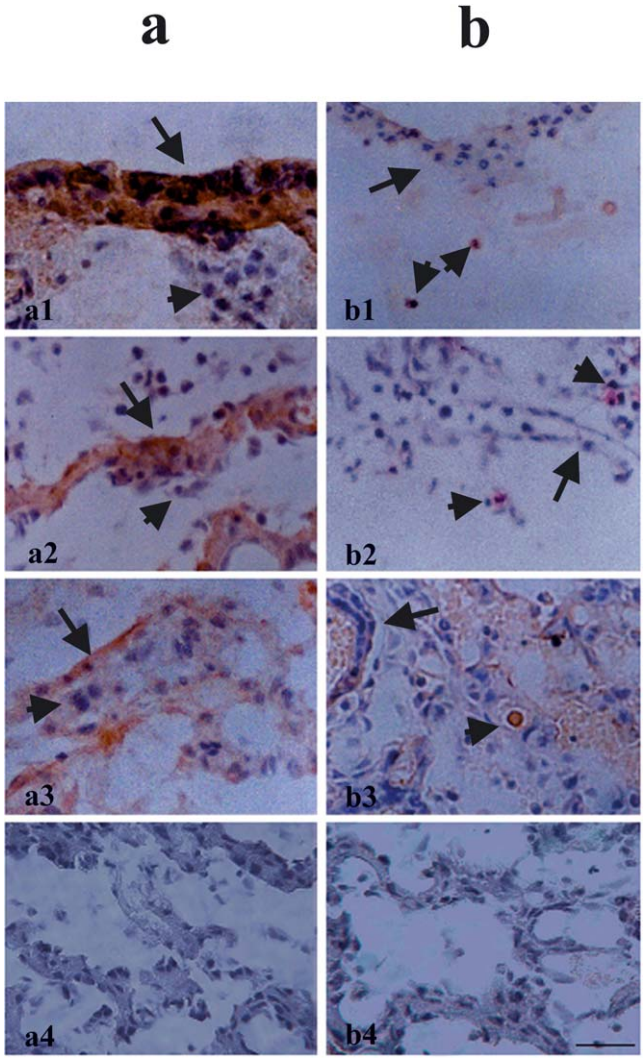

Fig. 2. Bcl-2 and Bax expression in g.d. 12 pregnant rabbit placenta. a: Bcl-2 expression. The stain was developed with 3,3′ diaminobenzidine and H_2O_2. Bcl-2 antibody clone C-2. (a1) In normal placenta (three cases), the staining was confined to the syncytiotrophoblast cytoplasm (long arrow), whereas the cytotrophoblast (short arrow) was negative. (a2) In placenta from rabbit treated with 50,000 IU IFNγ at g.d. 6 and killed at g.d. 12 (three cases), the staining was confined to the syncytiotrophoblast cytoplasm (long arrow), whereas the cytotrophoblast (short arrow) was negative. (a3) In placenta from rabbit treated with 100,000 IU IFNγ at g.d. 6 and killed at g.d. 12 (three cases), the staining was confined to the syncytiotrophoblast cytoplasm (long arrow), whereas the cytotrophoblast (short arrow) was negative. (a4) In negative control section of placenta, the Bcl-2 antibody was replaced by mouse Ig-G. b: Bax expression. The stain was developed with 3,3′ diaminobenzidine and H_2O_2. Bax antibody clone B-9. (b1) In normal placenta (three cases), the staining was confined to the limited cytotrophoblast cytoplasm (short arrow), whereas the syncytiotrophoblast (long arrow) was negative. (b2) In placenta from rabbit treated with 50,000 IU IFNγ at g.d. 6 and killed at g.d. 12 (three cases), the staining was confined to the limited cytotrophoblast cytoplasm (short arrow), whereas the syncytiotrophoblast (long arrow) was negative. (b3) In placenta from rabbit treated with 100,000 IU IFNγ at g.d. 6 and killed at g.d. 12 (three cases), the staining was confined to the limited cytotrophoblast cytoplasm (short arrow), whereas the syncytiotrophoblast (long arrow) was negative. (b4) In negative control section of placenta, the Bcl-2 antibody was replaced by mouse Ig-G. Bar = 40 μm and applies to (a1)–(a4), (b1)–(b3).

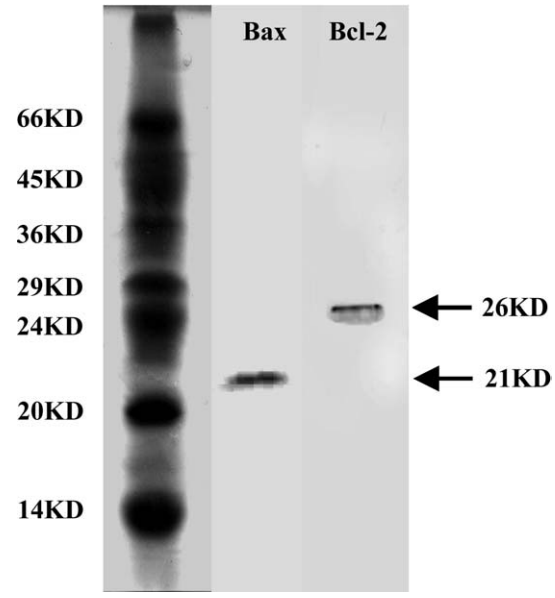

Fig. 3. Specific detection of antibodies of Bax and Bcl-2 by western blotting.

respectively, by 100,000 IU IFNγ administration. There is no significant difference in β-actin expression between control and IFNγ treated groups. These changes resulted in a 0.07 fold increase in the Bax to Bcl-2 ratio in 50,000 IU IFNγ administration and 0.41 fold increase in 100,000 IU IFNγ administration compared with those in the normal group (Fig. 4). There is significant difference in Bax to Bcl-2 ratio between control and 100,000 IU IFNγ treated groups ($P<0.05$).

3.3. Caspase-3 protein localization in placenta and the effect of IFNγ on its expression

Caspase-3 protein was localized to the syncytiotrophoblast and cytotrophoblast in placenta in normal and IFNγ treated groups (Fig. 5). IFNγ (50,000 IU) and 100,000 IU IFNγ increased caspase-3 expression 1.03 fold and 1.68 fold, respectively (Fig. 6). There is significant difference between control and 100,000 IU IFNγ treated groups ($P<0.05$).

4. Discussion

In the placenta, as in other complex organs, the development and maintenance of functional tissue structures depend on the balance among cellular proliferation, maturation and death. It has been reported that apoptosis plays an essential role in controlling the physiological cell kinetics in female reproductive organs and tissues, such as the endometrium [30], ovarian follicles [31], the regressing decidua [32] and the placenta. Dysregulation of the apoptotic process probably causes a variety of diseases, for example, enhanced apoptosis is

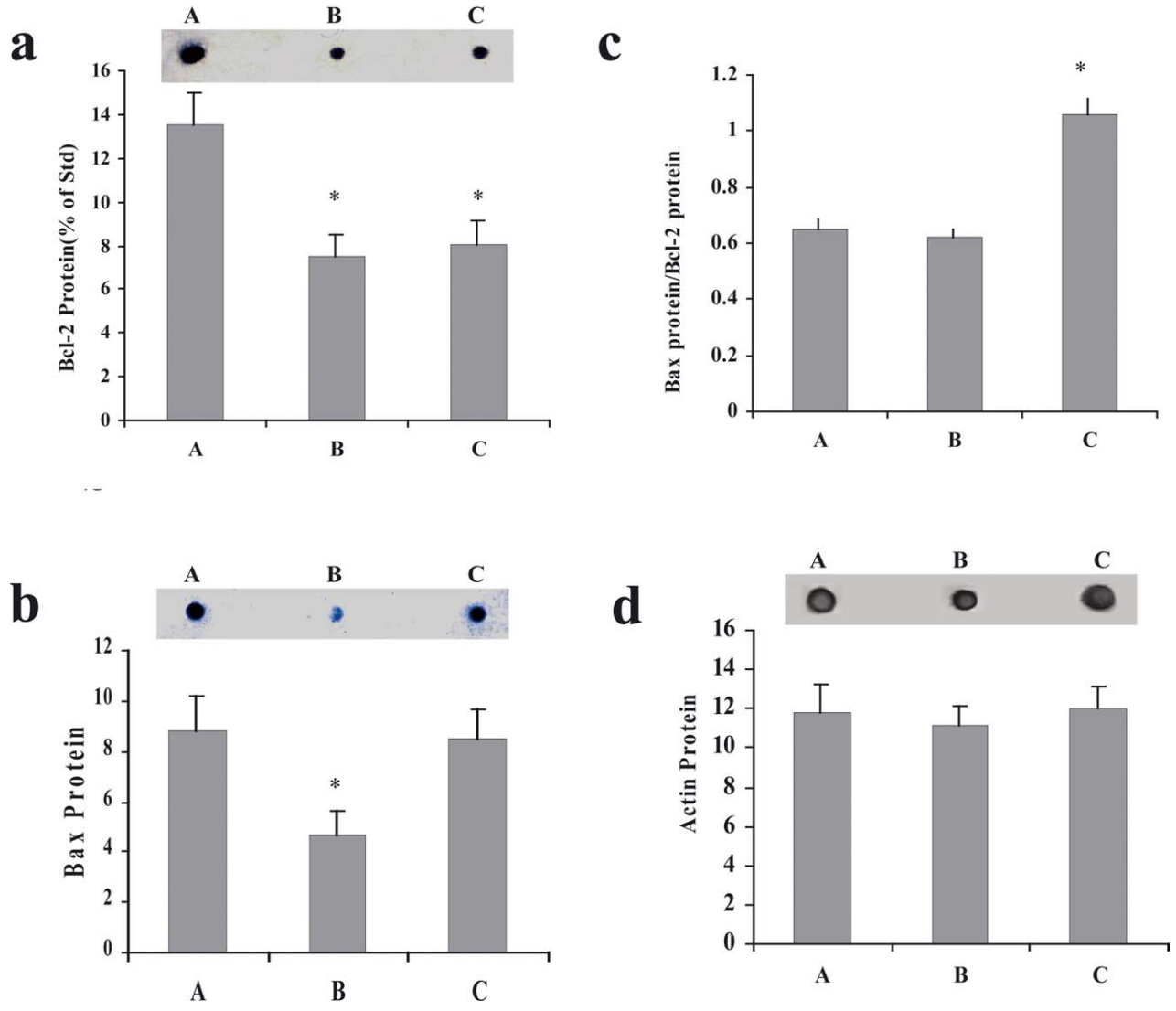

Fig. 4. Immunoblot analysis of Bcl-2 and Bax protein expression in placenta. (a) The upper panel is a representative immunoblot of Bcl-2 expression in placenta from control and IFNγ treated groups. The lower panel summarizes the densitometric analysis of replicate experiments ($n = 3$). Refer to Section 2 for detailed explanations. Statistical analysis was performed as in Fig. 1. *Means significant change compared with control groups ($P < 0.05$). A, control; B, treated with 50,000 IU IFNγ; C, treated with 100,000 IU IFNγ. (b) The upper panel is a representative immunoblot of Bax expression in placenta from control and IFNγ treated groups. The lower panel summarizes the densitometric analysis of replicate experiments ($n = 3$). Refer to Section 2 for detailed explanations. Statistical analysis was performed as in Fig. 1. *Means significant change compared with control groups ($P < 0.05$). A, control; B, treated with 50,000 IU IFNγ; C, treated with 100,000 IU IFNγ. (c) The effect of IFNγ on the Bax to Bcl-2 ratio. Statistical analysis was performed as in Fig. 1. There is significant change between control and 100,000 IU IFNγ treated groups ($P < 0.05$). (d) The upper panel is a representative immunoblot of β-actin expression in placenta from control and IFNγ treated groups. The lower panel summarizes the densitometric analysis of replicate experiments ($n = 3$). Refer to Section 2 for detailed explanations. Statistical analysis was performed as in Fig. 1. A, control; B, treated with 50,000 IU IFNγ; C, treated with 100,000 IU IFNγ.

associated with abnormal pregnancies such as first trimester abortions, ectopic pregnancies [17], fetal growth restriction or preeclampsia [33,34].

The present study investigated whether apoptosis in placenta was affected by IFNγ administration. DNA fragmentation analysis revealed the ladder pattern that is characteristic of the apoptotic cleavage of nuclear DNA in all cases and the small DNA fragments increased significantly in placenta treated with high dose of IFNγ. Our analysis in situ resulted in dense labelling of the syncytiotrophoblast in all cases. To date there are no reports about apoptosis in rabbit placenta and the effect of IFNγ on apoptosis in plancenta. The present study demonstrated that apoptosis occurs in normal rabbit placenta and IFNγ induced the extent of apoptosis for the first time. The results of apoptosis occurring in normal placenta from pregnant rabbit accorded with the previous work in human [15,16]. It is suggested that a possible benefit of the loss of trophoblast during gestation is consequential thinning.

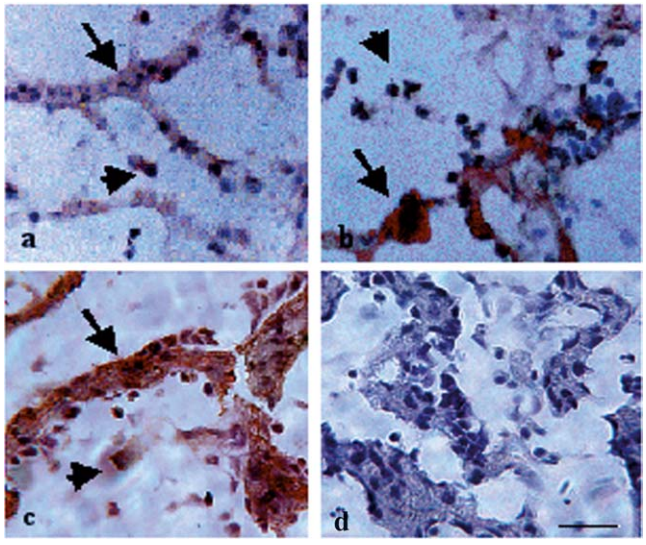

Fig. 5. Active caspase-3 localization. The stain was developed with 3,3′ diaminobenzidine and H_2O_2. Active caspase-3 antibody clone E-8. (a) In normal placenta (three cases), the staining was confined to the syncytiotrophoblast (long arrow) and cytotrophoblast cytoplasm (short arrow). (b) In placenta from rabbit treated with 50,000 IU IFNγ at g.d. 6 and killed at g.d. 12 (three cases), the staining was confined to the syncytiotrophoblast (long arrow) and cytotrophoblast cytoplasm (short arrow). (c) In placenta from rabbit treated with 100,000 IU IFNγ at g.d. 6 and killed at g.d. 12 (three cases), the staining was confined to the syncytiotrophoblast (long arrow) and cytotrophoblast cytoplasm (short arrow). (d) In negative control section of placenta, the caspase-3 antibody was replaced by mouse Ig-G. Bar = 40 μm and applies to (a)–(c).

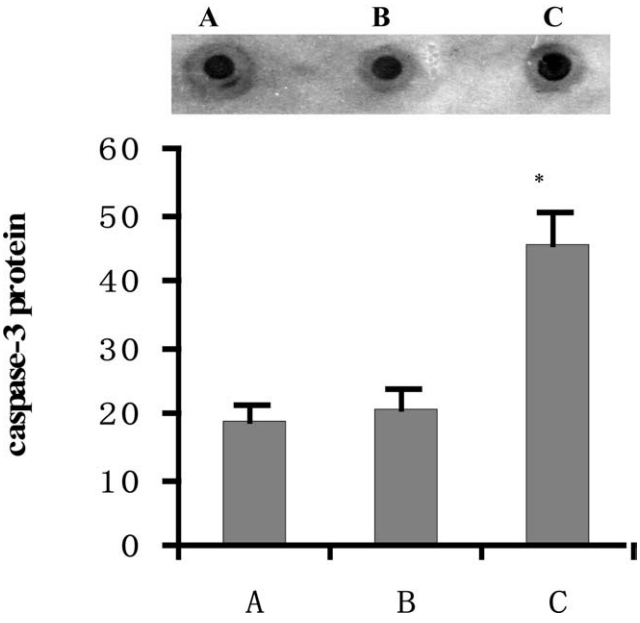

Fig. 6. Immunoblot analysis of active caspase-3. The upper panel is a representative immunoblot of active caspase-3 protein in placenta from control and IFNγ treated groups. The lower panel summarizes the densitometric analysis of replicate experiments ($n = 3$). Refer to Section 2 for detailed explanations. Statistical analysis was performed as in Fig. 1. There is significant change between control and 100,000 IU IFNγ treated groups ($P < 0.05$). A, control; B, treated with 50,000 IU IFNγ; C, treated with 100,000 IU IFNγ.

Trophoblast thickness is an important determinant of passive diffusive conductance and evidence from various sources shows that attenuation in vivo is a positive adaptive response [35,36].

In human placenta Nelson et al. [37] have assessed the expression of three important members of the bcl-2 gene family: Bax, Bcl-2 and Bak. Bcl-2 protein, which is known to inhibit apoptosis, was expressed throughout the syncytium of normal villi with much less staining in cytotrophoblast. Bcl-2 protein was least abundant in term placentas complicated by severe preeclampsia or intrauterine growth retardation than in normal term placentas [38]. Expression of the pro-apoptotic Bax protein was undetectable in the syncytiotrophoblast and was expressed in rare cytotrophoblast. Localization of a second pro-apoptotic protein, Bak, revealed immunoreactivity in isolated areas of intact syncytium of normal villi [37]. In the present study, we surveyed the changing patterns of Bax and Bcl-2 expression in normal placenta and IFNγ treated placenta using immunohistochemistry and immunoblot. Our analysis using immunohistochemistry indicated that syncytiotrophoblasts were stained for Bcl-2, whereas cytotrophoblasts were negative in normal and IFNγ treated placenta. The results agree with the work of Dai et al. [27].

There are different reports in the level of expression of bcl-2 and bax. Amato et al. [39] reported that Bcl-2 expression in human placenta was maximal during the second trimester while Bax protein was greatest during the first and third trimesters. Whereas, De Falco et al. [40] reported that Bcl-2 was generally expressed at low levels during the entire gestational period. On the other hand, Bax was low during the first trimester but increased towards the end of gestation. Our data suggested that the level of expression of both Bcl-2 and Bax was increased during the gestational period [41].

Baker et al. [42] reported TUNEL staining produced incongruous results with transmission electron microscopy, which remains the criterion standard in the assessment of apoptosis, so their findings discredit the use of TUNEL staining for the assessment of placental apoptosis. We think it is still a convenient method to detect apoptosis. To date in most studies the level of apoptosis is detected by the TUNEL method.

In accordance with the change of ratio of Bax to Bcl-2, apoptosis occurs in all placental cell types and increases from first to third trimester. The literature is almost consistent in this point of view [40,43].

More apparent apoptosis was found in the cytotrophoblasts and syncytiotrophoblasts layer of villi from pregnancies complicated by fetal growth restriction, preeclampsia or most failing pregnancies than in the trophoblast layer of villi from control pregnancies

[33,34]. There was no difference between the two groups in the expression of the proteins from the Bcl-2 family.

The result of our immunoblot assay showed that IFNγ decreased both Bcl-2 and Bax expression but increased the Bax to Bcl-2 ratio. The results provided the evidence that the Bax to Bcl-2 "rheostat" might be a more critical factor in regulating apoptosis in the IFNγ treated placenta than Bcl-2 and Bax's function in controlling cell death independently. These findings were in agreement with those of Tilly et al. [44] who reported that the ratio of Bcl-2 to Bax expression was probably a critical determinant of cell fate in ovarian granulosa cells during follicular maturation and atresia.

The Bax to Bcl-2 ratio is an important life and death rheostat for cells and this rheostat is influenced by competitive dimerization between the two proteins [45]. When there is an excess level of Bax, hetero-dimerization of Bax and Bcl-2 inhibits the action of Bcl-2 while homo-dimerization of Bax triggers the death signal cascade in the mitochondria. Bax homo-dimers destabilize the lipid bilayer structure of the outer mitochondrial membrane, promoting formation of a pore large enough to allow release of cytochrome *c* and apoptosis inducing factor (AIF) into the cytosol [46]. In the cytosol, cytochrome *c* binds to apoptotic protease activating factor-1 (Apaf-1) in the presence of dATP. Caspase-9 is then activated by binding of the caspase recruitment domain (CARD) in its prodomain to the CARD in Apaf-1. Then caspase-3 is activated and the apoptotic cells were executed.

Caspase-3 is one of effectors of apoptosis. It participates in apoptosis in a manner reminiscent of a well-planned and executed military operation. It cuts off contacts with surrounding cells, reorganizes the cytoskeleton, shuts down DNA replication and repair, interrupts splicing, destroys DNA, disrupts the nuclear structure, induces the cell to display signals that mark it for phagocytosis, and disintegrates the cell into apoptotic bodies. In the present study, caspase-3 localization was identified in syncytiotrophoblast and cytotrophoblasts, and showed increment in placenta with treatment of IFNγ.

Taking the previous work [41,47] and our present study together, we can conclude that the administration of IFNγ into pregnant rabbit at the time of implantation may induce apoptosis in syncytiotrophoblast within six days, and one of effects of IFNγ results from the enhancement of Bax to Bcl-2 ratio and the activated caspase-3. It suggested that the application of IFNγ to a pregnant female would result in dysregulation of apoptosis in placenta, which would lead to abortion.

References

[1] Hockenbery DM. Bcl-2 in cancer, development and apoptosis. J Cell Sci 1994;18(Suppl.):51–5.
[2] Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994;73:2013–26.
[3] Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994;124:1–6.
[4] Steller H. Mechanisms and genes of cellular suicide. Science 1995;267:1445–9.
[5] Yang E, Korsmeyer SJ. Molecular thanatopsis: a discourse on the Bcl-2 family and cell death. Blood 1996;88:386–401.
[6] Martimbeau S, Tilly JL. Physiological cell death in endocrine dependent tissues: an ovarian perspective. Clin Endocrinol 1997;46:241–54.
[7] Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609–19.
[8] Knudson CM, Korsmeyer SJ. Bcl-2 and Bax function independently to regulate cell death. Nat Genet 1997;16:358–63.
[9] Zha H, Reed JC. Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. J Biol Chem 1997;272(31):482–8.
[10] Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med 1997;3:614–20.
[11] Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326:1–16.
[12] Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell 1997;91:443–6.
[13] Nicholson DW, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci 1997;22:299–306.
[14] Huppertz B, Frank HG, Reister F, Kingdom J, Korr H, Kaufmann P. Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments in vitro. Lab Invest 1999;79(12):1687–702.
[15] Nelson DM. Apoptotic changes occur in syncytiotrophoblast of human placental villi where fibrin type fibrinoid is deposited at discontinuities in the villous trophoblast. Placenta 1996; 17:387–91.
[16] Smith SC, Baker PN, Symonds EM. Placental apoptosis in normal human pregnancy. Am J Obstet Gynecol 1997;177:57–65.
[17] Kokawa K, Shikone T, Nakano R. Apoptosis in human chorionic villi and decidua during normal embryonic development and spontaneous abortion in the first trimester. Placenta 1998; 19:21–6.
[18] Bocher WO, Herzog-Hauff S, Schlaak J, Meyer zum Buschenfeld KH, Lohr HF. Kinetics of hepatitis B surface antigen-specific immune responses in acute and chronic hepatitis B or after HBs vaccination: stimulation of the in vitro antibody response by interferon gamma. Hepatology 1999;29(1):238–44.
[19] Dallalio G, North M, Means RT. Inhibition of marrow CFU-E colony formation from human immunodeficiency virus-infected patients by beta- and gamma-interferon. Am J Hematol 1996;53(2):118–20.
[20] Schneider A, Grubert T, Kirchmayr R, Wagner D, Papendick U, Schlunck G. Efficacy trial of topically administered interferon gamma-1 beta gel in comparison to laser treatment in cervical intraepithelial neoplasia. Arch Gynecol Obstet 1995;256(2):75–83.
[21] Haddad EK, Duclos AJ, Antecka E, Lapp WS, Bains MG. Role of interferon-gamma in the priming of decidual macrophages for nitric oxide production and early pregnancy loss. Cell Immunol 1997;181(1):68–75.
[22] Chaouat GE, Menu DA, Clark MD, Mindowski M, Wegmann TG. Control of fetal survival in CBA × DBA/2 mice by lymphokine therapy. J Reprod Fertil 1994;89:447.
[23] Cao Y, Sun D, Chen Y, Zhu P. Studies on the antifertility effect and the mechanism of human recombinant interferon-gamma in rabbits. J Reprod Med 1999;8(2):98–102.
[24] Yui J, Garcia-Lloret MI, Wegmann TG, Guilbert LJ. Cytotoxicity of tumor necrosis factor alpha and gamma-interferon against primary human placental trophoblasts. Placenta 1994;15:819–25.

[25] Trubiani O, Bosco D, Di Primio R. Interferon-gamma (IFN-gamma) induces programmed cell death in differentiated human leukemic B cell lines. Exp Cell Res 1994;215(1):23–7.
[26] Blin N, Stafford DW. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res 1976;3:2303.
[27] Dai D, Moulton BC, Ogle TF. Regression of the decidualized mesometrium and decidual cell apoptosis are associated with a shift in expression of Bcl-2 family members. Biol Reprod 2000;63:188–95.
[28] Smit V, van Veelen PA, Tjaden UR, van dev Geef J, Haaijman JJ. Human interleukin-3 contains a discontinuous zinc binding domain. Biochem Biophys Res Commun 1992;187(2):859–66.
[29] Ishihara M, Ishida T, Isogai E, Kimura K, Oritsu M, Matsui Y, et al. Detection of antibodies to *Borrelia* species among patients with confirmed sarcoidosis in a region where Lyme disease is non-endemic. Graefes Arch Clin Exp Ophthalmol 1996;234(12):770–3.
[30] Gompel A, Sabourin JC, Martin A, Yaneva H, Audouin J, Decroix Y, et al. Bcl-2 expression in normal endometrium during the menstrual cycle. Am J Pathol 1994;144:1195–202.
[31] Tilly JL. Use of terminal transferase DNA labeling reaction for the biochemical and in situ analysis of apoptosis. In: Celis JE, editor. Cell biology: a laboratory handbook. San Diego: Academic Press; 1994. p. 330–7.
[32] Gu Y, Jow GM, Moulton BC, Lee C, Sensibar JA, Park-Sarge OK, et al. Apoptosis in decidual tissue repression and reorganization. Endocrinology 1994;135:1272–9.
[33] Levy R, Smith SD, Yusuf K, Huettner PC, Kraus FT, Sadovsky Y, et al. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. Am J Obstet Gynecol 2002;186(5):1056–61.
[34] Allaire AD, Ballenger KA, Wells SR, McMahon MJ, Lessey BA. Placental apoptosis in preeclampsia. Obstet Gynecol 2000; 96(2):271–6.
[35] Mayhew TM, Jackson MR, Haas JD. Microscopical morphology of the human placenta and its effects on oxygen diffusion: a morphometric model. Placenta 1986;7:121–31.
[36] Mayhew TM, Burton GJ. Stereology and its impact on our understanding of human placental functional morphology. Microsc Res Tech 1997;38:195–205.
[37] Ratts VS, Tao XJ, Webster CB, Swanson PE, Smith SD, Brownbill P, et al. Expression of BCL-2, BAX and BAK in the trophoblast layer of the term human placenta: a unique model of apoptosis within a syncytium. Placenta 2000; 21(4):361–6.
[38] Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez J, Samoto T, Maruo T. Changes in proliferative potential, apoptosis and Bcl-2 protein expression in cytotrophoblasts and syncytiotrophoblast in human placenta over the course of pregnancy. Endocr J 2000;47(3):317–27.
[39] Amato P, Zwain I, Reed J, Yen SSC. Expression of Bcl-2 and Bax in human placenta. J Soc Gynecol Investig 1996;3(Suppl. 2).
[40] De Falco M, De Luca L, Acanfora F, Cavallotti I, Cottone G, Laforgia V, et al. Alteration of the Bcl-2 : Bax ratio in the placenta as pregnancy proceeds. Histochem J 2001;33(7):421–5.
[41] Liu Z, Chen Y, Yang Y, Peng J-P. Change of apoptosis and apoptotic relative gene Bcl-2 and Bax expression in pregnant rabbit placenta. Prog Biochem Biophys 2002;29(1):64–8.
[42] Smith SC, Leung TN, To KF, Baker PN. Apoptosis is a rare event in first-trimester placental tissue. Am J Obstet Gynecol 2000; 183(3):697–9.
[43] Lea RG, Riley SC, Antipatis C, Hannah L, Ashworth CJ, Clark DA, et al. Cytokines and the regulation of apoptosis in reproductive tissues: a review. Am J Reprod Immunol 1999; 42(2):100–9.
[44] Tilly JL, Tiley KI, Kenton ML, Jonson AL. Expression of members of the bcl-2 gene family in immature rat ovary: equine chorionic gonadotropin medicated inhibition of granulosa cell apoptosis is associated with decrease bax and continuative bcl-2 and bcl-x long messenger ribonucleic acid levels. Endocrinology 1995;136:232–41.
[45] Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322–6.
[46] Basanez G, Nechushtan A, Drozhinin O, Chanturiya A, Choe E, Tutt S, et al. Bax, but not Bcl-XL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc Natl Acad Sci U S A 1999;96:5492–7.
[47] Liu Z, Chen Y, Yang Y, Peng J-P. The effect on MHC class II expression and apoptosis in placenta by IFNγ administration. Contraception 2002;65:177–84.