

P R A C E O R Y G I N A L N E

położnictwo

# Expression of caspase-3, Bax nad Bcl-2 in placentas from pregnancies complicated by treated and non-treated fetal growth restriction

Ekspresja kaspazy-3, Bax i Bcl-2 w łożyskach ciąż leczonych i nieleczonych z powodu wewnątrzmacicznego zahamowania wzrastania płodu

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## Abstract

**Background:** Fetal growth restriction (FGR) is the reason of high prematurity rate and its later complications. Restriction of utero-placental circulation, which could be changed by IURG treatment, plays the main role in FGR. The results of changes in apoptosis-related genes expression due to FGR treatment may help further in the prevention and treatment of FGR.

**Material and methods:** Caspase-3, Bax and Bcl-2 expressions in normal pregnancies and those complicated by treated and untreated FGR have been compared. The study was conducted in 2005-2006 at the High-Risk Pregnancy Unit of Medical University in Łódź and Kopernik Hospital in Łódź.

Caspase-3, Bax and Bcl-2 expressions were assessed by immunohistochemical method. Bcl-2 was assessed in the trophoblast, Bax and caspase-3 in the decidua and the trophoblast.

**Results:** The mean value of Bcl-2 in the trophoblast was  $58.8 \pm 12.7$  in the FGR-untreated group,  $37.0 \pm 0.5$  in the FGR-treated group and  $65.7 \pm 6.9$  in the control group.

In the FGR-untreated group the mean value of Bax expression was  $60.6 \pm 10.7$  in the trophoblast and  $32.0 \pm 7.3$  in the decidua. In the FGR-treated group the mean value of Bax expression was  $42.2 \pm 12.2$  in the trophoblast and  $20.9 \pm 6.4$  in the decidua. In the control group the mean value of Bax expression was  $13.6 \pm 2.2$  in the trophoblast and  $6.6 \pm 6.8$  in the decidua.

In the FGR-untreated group the mean value of Cpp-32 expression was  $40.1 \pm 9.1$  in the trophoblast and  $42.6 \pm 12.5$  in the decidua. In the FGR-treated group the mean value of Cpp-32 expression was  $21.3 \pm 6.8$  in the trophoblast and  $23.7 \pm 5.1$  in the decidua. In the control group the mean value of Cpp-32 expression was  $13.6 \pm 6.3$  in trophoblast and  $11.6 \pm 5.3$  in the decidua.

**Conclusions:** Increased expression of pro-apoptotic proteins in the placenta might be one of the reasons for FGR development. The treatment used in the FGR group decreased the process of apoptosis.

Key words: **apoptosis / caspase-3 / Bax / Bcl-2 / fetal growth retardation / placenta / IURG treatment /**

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## Streszczenie

Hipotrofia wewnątrzmaciczna (FGR) wiąże się z wysokim odsetkiem wcześniactwa i jego późniejszych powikłań. W patogenezie hipotrofii główną rolę odgrywa ograniczenie przepływu maciczno-łożyskowego, co może być modyfikowane przez stosowanie terapii. Uzyskanie zmian zachodzących podczas leczenia w zakresie procesu apoptozy może wpłynąć w przyszłości na sposób zapobiegania i leczenia hipotrofii wewnątrzmacicznej.

**Materiał i metody:** Porównano ekspresję kaspazy-3, Bax i Bcl-2 w ciąży niepowikłanej i powikłanej hipotrofią wewnątrzmaciczną z podziałem na kobiety nieleczone i leczone. Badania były prowadzone w Klinice Patologii Ciąży Uniwersytetu Medycznego w Łodzi w latach 2005-2006. Ekspresję kaspazy-3, Bax i Bcl-2 oceniano metodami immunohistochemicznymi. Białko Bcl-2 było oceniane w trofoblaście, Bax i kaspaza-3 w doczesnej i trofoblaście.

**Wyniki:** W grupie nieleczonych średnia wartość ekspresji Bcl-2 w trofoblaście wynosiła,  $58,8 \pm 12,7$ , w grupie leczonych  $37,0 \pm 10,5$ , w grupie kontrolnej  $65,7 \pm 6,9$ . W grupie nieleczonych średnia wartość Bax w trofoblaście wynosiła  $60,6 \pm 10,7$ , w doczesnej natomiast  $32,0 \pm 7,3$ . W grupie kobiet leczonych średnia wartość ekspresji w trofoblaście wynosiła  $42,2 \pm 12,2$ , w doczesnej natomiast  $20,9 \pm 6,4$ . W grupie kontrolnej w trofoblaście wynosiła  $13,6 \pm 2,2$ , a w doczesnej  $6,6 \pm 6,8$ . W grupie nieleczonych średnia wartość kaspazy-3 mierzonej za pomocą aktywności Cpp-32 wynosiła w trofoblaście  $40,1 \pm 9,1$ , a w doczesnej  $42,6 \pm 12,5$ . W grupie kobiet leczonych średnia ekspresja Cpp-32 w trofoblaście wynosiła  $21,3 \pm 6,8$ , w doczesnej  $23,7 \pm 5,1$ . W grupie kontrolnej w trofoblaście wartość ekspresji Cpp-32 oceniano na  $13,6 \pm 6,3$ , w doczesnej uzyskano wartości  $11,6 \pm 5,3$ .

**Wnioski:** Podwyższona ekspresja białek proapoptotycznych w łożysku może być jednym z powodów rozwoju hipotrofii wewnątrzmacicznej. Leczenie zastosowane w hipotrofii wewnątrzmacicznej obniżyło oceniane parametry apoptozy.

Słowa kluczowe: **apoptoza / kaspaza-3 / bax / bcl-2 / hipotrofia wewnątrzmaciczna / łożysko / leczenie /**

## Introduction

Intrauterine fetal growth restriction (FGR) means a newborn body mass less than the 10<sup>th</sup> centile with respect to the gestational age. Early and precise diagnosis of FGR is based on the ultrasound biometric evaluation of the fetus compared with the results obtained in the early pregnancy. The cases that started early, before the 24<sup>th</sup> week of pregnancy, are more serious, with less favorable prognosis. FGR may depend on various reasons, but more than 80% cases of FGR of unclear origin seem to be connected with changes in the utero-placental circulation and blood vessels malfunction.

There are many factors influencing the blood flow in the utero-placental and fetal circulation, chief among them an increased activity of free radicals. An increased apoptosis is observed in many processes connected with increased free radicals activity.

In our previous studies on free radical reactions in FGR and normal pregnancies the results of fetal growth and Doppler flows in the umbilical and mild cerebral arteries confirmed the safety and effectiveness of the applied pharmacological treatment.

The standard treatment of ultrasound-diagnosed FGR in the Clinic of High Risk Pregnancy was based on L-arginine and acetylsalicylic acid.

L-arginine is a nitric oxide (NO) precursor which has a strong diastolic effect on the vessels. The endothelial nitric oxide synthase, the content of the e-NOS enzyme, is thought to regulate NO synthesis in blood vessels [1].

Acetylsalicylic acid affects the inhibition of the thromboxane synthase, and by this decreases the synthesis of thromboxane. The increase of prostacyclin concentration and decrease of thromboxane results in relaxation of the vessels, inhibits the activity of platelets cyclooxygenase and has an anti-aggregative effect on the platelets and improves the utero-placental circulation.

Programmed cell death, apoptosis, which determines cell death is regulated by independent, multifactor, often contradictory processes [2].

In the group of Bcl proteins we can find two ways of action: pro- and anti-apoptotic. Anti-apoptotic proteins of Bcl-2 group decrease apoptosis by decreasing cytochrome C release and blocking caspase-9 activity. This action could protect mitochondrial membranes. Caspase-9 is one of the enzymes from protease group, initiating apoptosis - cysteine proteinase. Initiating factors and specific effectors of caspases complex compose the death initiation signaling complex (DISC).

Bax, the protein from the Bcl family, has a different, pro-apoptotic action. It is activated by Bid protein and decreases mitochondrial membrane permeability [3].

Caspase-3 – CPP32, apopain, is one of the main effectors of apoptosis reactive downstream of caspase-9 apoptotic pathway. In cells caspase-3 is present as an inactive proenzyme and could be activated by proteolytic process to its active subunits when cells undergo apoptosis. Some data suggests that caspase-3 is an important mediator of apoptosis in the immune system and during the morphogenetic cell death in the mammalian brain [4, 5].

Increased apoptosis can restrict the utero-placental exchange and affect fetal growth restriction.

## Aim

The aim of this study was to find the difference in apoptotic activity expressed by the activity of caspase-3, Bcl-2 and Bax in placentas from normal pregnancies and from those complicated by fetal growth restriction – both, untreated and pharmacologically treated.

Expression of caspase-3, Bax nad Bcl-2 in placentas from pregnancies complicated by treated and non-treated fetal growth restriction.

## Materials and Methods

The study was conducted in 2006-2007 at the High Risk Pregnancy Clinic of Medical University of Łódź. A written informed consent of all subjects and the written approval of University Ethics Committee were obtained.

Three groups of patients were compared: 1<sup>st</sup> group: pregnant women in pregnancy complicated by fetal growth restriction diagnosed postnatally – the weight of the newborns in centile charts 9-5: 20 cases. 2<sup>nd</sup> group: pregnant women in pregnancy complicated by fetal growth restriction diagnosed prenatally - treated by L-arginine and acetylsalicylic acid: 43 cases. All cases of FGR were of unknown causes. 3<sup>rd</sup> group: controls, pregnant women with normal neonatal weight: 15 cases.

2x2 excision from the central part of the placenta, near the umbilical cord insertion, was taken in aseptic conditions, fixed in the formalin solution and absorbed in paraffin blocks.

Bcl-2, anti-apoptotic protein was assessed by an immunohistochemical method by monoclonal mouse antibodies anti human Bcl-2 oncoprotein. Bax rabbit anti human polyclonal antibody was used to assess Bax activity. To evaluate the activity of caspase-3 polyclonal rabbit antibodies anti human Cpp-32 protein was used. All antibodies were produced by Dakocytomation.

Bcl-2 was assessed in the trophoblast. The assessment of Bax and caspase-3 was done in two regions of placental fragments: the decidua and the trophoblast.

A statistical analysis was done using non-parametric U-Mann-Whitney and t-Student's tests. p value <0.05 was considered statistically significant.

## Results

The assessment of Bcl-2 was done in the trophoblast by counting brown points in ten random zones of vision. In the group of untreated intrauterine growth restriction (20 cases), the lowest mean expression was 23.8 the highest 53.7, the mean expression of Bcl-2 was 37.0±10.5.

In the group of intrauterine growth restriction treated by L-arginine the evaluation was done in 43 fragments. The lowest mean expression of Bcl-2 was 30.0, the highest 73.7 and the mean value was 58.8±12.7. In the control group the evaluation of Bcl-2 value was done in 15 fragments of placentas in ten random zones of vision. The lowest mean expression of Bcl-2 was 53.7, the highest 75.7 and the mean value was 65.74±6.9. (Table I).

In a group of healthy mothers of normal-weight newborns the expression of Bcl-2 was higher than in both FGR groups. In the FGR-untreated group it was lower than in the FGR-treated group. The difference between Bcl-2 expression in the compared groups was statistically significant (p<0.05).

The evaluation of Bax was done in the trophoblast and the decidua separately, by counting brown points in ten and six random zones of vision respectively. The lowest mean expression of Bax in FGR-untreated group in the trophoblast was 28.0, the highest was 70.1, the mean value was 60.57±10.73. In the FGR-treated group, the lowest mean expression of Bax in the trophoblast was 23.5, the highest was 68.8 and the mean value was 42.2±12.2. In the control group in the trophoblast the lowest mean expression of Bax was 10.4, the highest was 16.3, the mean value was 13.6±2.2. (Table II).

In a group of healthy mothers of normal-weight newborns the expression of Bax in the trophoblast was lower than in both

FGR groups. In the FGR-untreated group the expression of Bax was higher than in group of pharmacologically treated FGR. The difference between Bax expression in the trophoblast in the compared groups was statistically significant (p<0.05).

In the decidua part of the placenta in the FGR-untreated group the lowest mean expression of Bax was 20.0, the highest was 45.5 and the mean value was 32.0±7.3. In the FGR-treated group the lowest mean expression of Bax was 9.8, the highest was 40.7 and the mean value was 20.9±6.4. In the control group the lowest mean expression of Bax was 2.3, the highest was 24.0 and the mean value was 6.6±6.8. (Table III).

In both areas, the trophoblast and the deciduas, the expression of Bax was significantly higher in the placentas from FGR-complicated pregnancies than in the control group (p<0.05).

Caspase-3 measurement showed as Cpp-32 expression was done in two regions: the trophoblast and the decidua.

In the FGR-untreated group the lowest mean expression of Cpp-32 in the trophoblast was 29.0 the highest was 59.5, the mean value was 40.1±9.1. In the group of intrauterine growth restriction treated by L-arginine the lowest mean expression of Cpp-32 was 12.8, the highest was 39.8 and the mean value was 21.3±6.8.

In the control group the lowest mean expression of Cpp-32 was 7.0, the highest was 25.6 and the mean value was 13.6±6.3. (Table IV).

In the FGR-untreated group the lowest mean expression of Cpp-32 in the decidua was 17.5, the highest was 65.5 and the mean value was 42.6±12.5. In the group of intrauterine growth restriction treated by L-arginine the lowest mean expression of Cpp-32 was 12.2, the highest was 30.5 and the mean value was 23.7±5.1.

In the control group the lowest mean expression of Cpp-32 was 4.3, the highest was 20.8 and the mean value was 11.6±5.3.

In the control group the expression of Cpp-32 was higher than in both FGR groups. In the FGR-untreated group the expression of Cpp-32 was lower than in the FGR-treated one. The difference between Cpp-32 expression in every compared group was statistically significant (p<0.05). (Table V).

## Discussion

In our study the etiology of FGR was unknown and such inductive factors as cigarette smoking, hypertension, genetic disorders, infections in early pregnancy, cholestasis, heart and urinal tract diseases were excluded, owing to which our groups of patients were very similar. In many studies on fetal growth restriction those factors were present and could have changed the results.

The treatment used in our study makes significant changes on values of selected pro- and anti-apoptotic factors in the group of FGR mothers. As we observed, the treatment of FGR decreases metabolites of free radicals reactions. Those metabolites, which have restrictive action on blood vessels, cause the decrease of mother-to-fetus exchange and may initiate FGR in pregnancy. An improvement of blood circulation leads to the decrease of free radicals reactions and to the reduction of small vessels restriction.

Some of the parameters of lipid peroxidation process cause destruction of the mitochondrial membranes in the central nervous system and of other tissues, what can also cause cell destabilization.

**Table I.** Bcl-2 expression in the trophoblast in the compared groups (values from 10 zones of vision).

| Group          | N  | Min  | Max  | Mean | SD   | SEM | groups | p      |
|----------------|----|------|------|------|------|-----|--------|--------|
| Untreated (G1) | 20 | 23.8 | 53.7 | 37.0 | 10.5 | 2.4 | G1-G2  | <0.001 |
| Treated (G2)   | 43 | 30.0 | 73.7 | 58.8 | 12.7 | 2.0 | G1-G3  | <0.001 |
| Control (G3)   | 15 | 53.7 | 75.7 | 65.7 | 7.0  | 2.2 | G2-G3  | Ns     |

**Table II.** Bax expression in the trophoblast in the compared groups.

| Group          | N  | Min  | Max  | Mean | SD   | SEM | groups | p      |
|----------------|----|------|------|------|------|-----|--------|--------|
| Untreated (G1) | 20 | 28.0 | 70.1 | 60.2 | 10.5 | 2.5 | G1-G2  | <0.001 |
| Treated (G2)   | 43 | 23.5 | 68.8 | 42.2 | 12.2 | 2.0 | G1-G3  | <0.001 |
| Control (G3)   | 15 | 8.0  | 16.3 | 12.5 | 2.9  | 1.0 | G2-G3  | <0.001 |

**Table III.** Bax expression in decidua in compared groups.

| Group         | N  | Min  | Max  | Mean | SD  | SEM | groups | p      |
|---------------|----|------|------|------|-----|-----|--------|--------|
| Untreated(G1) | 20 | 20.0 | 45.5 | 32.0 | 7.3 | 1.7 | G1-G2  | <0.001 |
| Treated (G2)  | 43 | 9.8  | 40.7 | 21.0 | 6.4 | 1.0 | G1-G3  | <0.001 |
| Control (G3)  | 15 | 2.3  | 24.0 | 6.6  | 6.8 | 2.3 | G2-G3  | <0.001 |

**Table IV.** Cpp-32 expression in the trophoblast in the compared groups.

| Group         | N  | Min  | Max  | Mean | SD  | SEM | groups | p      |
|---------------|----|------|------|------|-----|-----|--------|--------|
| Untreated(G1) | 20 | 29.0 | 59.5 | 40.1 | 9.1 | 2.0 | G1-G2  | <0.001 |
| Treated (G2)  | 40 | 12.8 | 39.8 | 21.3 | 6.8 | 1.1 | G1-G3  | <0.001 |
| Control (G3)  | 11 | 7.0  | 25.6 | 13.6 | 6.3 | 1.9 | G2-G3  | <0.001 |

**Table V.** Cpp-32 expression in the decidua in the compared groups.

| Group          | N  | Min  | Max  | Mean | SD   | SEM | groups | p      |
|----------------|----|------|------|------|------|-----|--------|--------|
| Untreated (G1) | 20 | 17.5 | 65.5 | 42.6 | 12.5 | 2.8 | G1-G2  | <0.001 |
| Treated (G2)   | 40 | 12.2 | 30.5 | 23.7 | 5.1  | 0.8 | G1-G3  | <0.001 |
| Control (G3)   | 11 | 4.3  | 20.8 | 11.6 | 5.3  | 1.6 | G2-G3  | <0.001 |

The results of L-arginine treatment show the decrease of elevated concentrations of HNE in the group of FGR pregnancies after the applied treatment. HNE, one of the toxic products of lipid peroxidation, is also involved in the modification of Cyt C and activity of caspases-system in the process of apoptosis. Increased concentration of HNE can also cause DNA fragmentation and be the reason of increased apoptosis [7].

In both groups of pregnancies complicated by fetal growth restriction we found higher expression of Bax than in the control group, that means higher activity of the apoptotic process. The importance of apoptosis cascade for the normal function of the trophoblast is a well-known fact. Apoptotic changes in the trophoblast have been described in many complications of pregnancy [8].

In our study in the decidual part of the placenta, apoptosis seems to be higher in the group of fetal growth restriction than in placentas from the control group, which is similar to the results of other authors [7].

Some of the pro-apoptotic proteins are highly expressed in human placenta with placental dysfunction as hypoxia and fetal growth restriction. It has been stated that placental hypoxia causes an increase of apoptosis, that could be the unknown, early factor for intrauterine growth restriction [8]. The changes in regulation of apoptosis in the extravillous trophoblast could cause restriction of blood flow and decrease the maternal-fetal circulation [4].

An increased apoptosis in spiral arteries that limits the trophoblast invasion into the decidua could be the reason for hypertension in pregnancy and FGR [6]. The trophoblast cells susceptibility to undergo apoptosis determines the apoptosis of the endovascular trophoblast and probably influences the transformation of the uterine spiral arteries [10].

We found high expression of Bax and low of Bcl-2 in the extravillous trophoblast, what is similar to the results obtained by Huppertz [5] and could be responsible for the malfunction of the vessels.

Our results are in disagreement with the observations of Endo, who found no significant difference in Bax expression in the normal and FGR placentas [9]. In our study in both regions, the trophoblast and the decidua, the expression of Bax in FGR group was higher than in the control group. High Bax expression showed in our study points to the role of increased apoptosis in placentas from fetal growth restriction.

The analysis of presence of apoptotic cells done in fetal membranes showed the increase of apoptosis in FGR group when compared to normal pregnancy [11].

In fetal growth restriction the density of microvilli was reduced and the basal membrane was thicker in FGR than in normal growth, what means that increased apoptosis in the syncytiotrophoblast which could cause growth restriction by cell deletion [12]. Our data show higher expression of estimated pro-apoptotic factors in both regions, the trophoblast and the decidua, in FGR group, what is similar to the observations of other authors [9, 10, 11, 12].

Immunohistochemical analysis shows significantly higher expression of the active form of caspase-3 in FGR than in a normal pregnancy in data presented by Endo [9].

Our study shows similar results of caspase-3 expression in both regions of placenta, the trophoblast and the deciduas. We have observed the highest expression of caspase-3 in the group of

untreated fetal growth restriction, lowering due to the L-arginine treatment. The lowest expression of caspase-3 was observed in the control group. This observation suggests that apoptosis through activation caspase-3 may play a role in FGR.

In our study one of the FGR therapy elements was acetylsalicylic acid and the results showing low expression of pro-apoptotic parameters in the FGR-treated group could be related not only to L-arginine but also to acetylsalicylic acid action.

Hills suggests the ability of another anticoagulant, heparin, to antagonize cell death process by activation of multiple anti-apoptotic pathways in the placenta to be potentially useful in the management of at-risk patients, even in the absence of thrombophilic disorder [13].

In the placentas of smoking mothers with growth-restricted infants apoptosis was also increased. The reduction in blood flow could also increase apoptosis, and it is possible that this could be one of the mechanisms playing a role in the growth restriction. In our study all pregnant women were non-smokers and the changes in the expression of pro-apoptotic factors observed in placentas were similar [14].

## Conclusions

1. Increased expression of pro-apoptotic proteins in the placenta might be one of the reasons for FGR development.
2. The treatment used in the FGR group decreased the process of apoptosis.

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