Maternal Hypothyroidism and Its Role in the Placenta: A Morphometric and Immunohistochemical Study

Maternal Hipotiroidizm ve Plasentadaki Rolü: Morfometrik ve İmmünohistokimyasal Çalışma

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ABSTRACT Objective: The aim of this study was to investigate the structure and mechanism of apoptosis in placentas with maternal hypothyroidism. **Material and Methods:** Seven normal and eight hypothyroidic mothers were selected. Tissues were prepared for histochemistry, immunohistochemistry and TUNEL assay for detection of apoptosis and structural changes. **Results:** Increased TUNEL-positive staining in the cytotrophoblast, syncytiotrophoblast and mesenchymal cells was shown in the placentas of the hypothyroid group in comparison to the control group. In the control group, positive immunostaining for Caspase-3 was moderate in the syncytiotrophoblast cells, while it was mild in the cytotrophoblast cells and it was negative in the mesenchymal cells. Immunostaining for Bcl-2 was moderate in the syncytiotrophoblast, cytotrophoblast and mesenchymal cells. Bax immunostaining was negative in the cytotrophoblast and mesenchymal cells, while immunostaining was mild in the syncytiotrophoblast. In the hypothyroid group, Caspase-3 immunostaining was strong in the syncytiotrophoblast, cytotrophoblast and mesenchymal cells, whereas Bcl-2 immunostaining was absent in the cytotrophoblasts, and mild in the syncytiotrophoblasts and mesenchymal cells. Bax immunostaining was moderate in the syncytiotrophoblasts and cytotrophoblasts, however it was mild in the mesenchymal cells. The mean number of syncytial knots was significantly lower in the control group than the hypothyroid group ($p< 0.05$). Mean thickness of medium size blood vessels was significantly lower in the hypothyroid group than the control group ($p< 0.05$). Mean area of stromal fibrosis demonstrated in the hypothyroid group was higher than the control group ($p< 0.05$). **Conclusion:** We conclude that significant histological changes occur in the placentas of hypothyroid mothers with associated high incidence of apoptotic marker response.

Key Words: Hypothyroidism; apoptosis; placenta; histology

ÖZET Amaç: Bu çalışmanın amacı, maternal hipotiroidizimli plasentaların yapısını ve apoptozisin mekanizmasını araştırmaktır. **Gereç ve Yöntemler:** Bu amaçla yedi normal ve sekiz hipotiroidik anne seçildi. Dokular apoptozisi ve yapısal değişiklikleri araştırmak üzere histokimya, immünohistokimya ve TUNEL işaretleme için hazırlandı. **Bulgular:** Hipotiroidik grup plasentalar kontrol grubu plasentalar ile karşılaştırıldığında TUNEL pozitif sitotrofoblast, sinsityotrofoblast ve mezenşimal hücre sayısının arttığı belirlendi. Kontrol grubuna ait plasentalarda, Kaspaz-3 işaretlemede sinsityotrofoblastlarda orta, sitotrofoblastlarda zayıf, mezenşimal hücrelerde negatif immün boyanma gözlendi. Bcl-2 işaretlemede sitotrofoblast, sinsityotrofoblast ve mezenşimal hücrelerde orta, Bax işaretlemede ise sinsityotrofoblastlarda zayıf, sitotrofoblast ve mezenşimal hücrelerde negatif immünboyanma gözlendi. Hipotiroidik grupta, Kaspaz-3 ile sitotrofoblast, sinsityotrofoblast ve mezenşimal hücreler kuvvetli tutulum gösterirken Bcl-2 ile işaretlemede sitotrofoblastlarda negatif, sinsityotrofoblast ve mezenşimal hücrelerde zayıf tutulum izlendi. Bax immun işaretlemede ise sitotrofoblast ve sinsityotrofoblastlarda orta, mezenşimal hücrelerde zayıf tutulum izlendi. Sinsityal tomurcukların ortalama sayısı normal plasentalarda anlamlı olarak düşüktü ($p<0.05$). Hipotiroidik grupta orta büyüklükteki damar duvarı ortalama kalınlığı normal grup plasentalarla karşılaştırıldığında anlamlı olarak düşüktü ($p< 0.05$). Hipotiroidik grupta kontrol grubuna göre stromal fibrozis artmıştı.($p< 0.05$). **Sonuç:** Apopitotik markırlara olan yanıtın yüksek olmasına bağlı olarak hipotiroidik annelerin plasentalarında anlamlı histolojik değişiklikler gözlenmektedir.

Anahtar Kelimeler: Hipotiroidizm; apopitozis; plasenta; histoloji

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The human placenta performs numerous functions during its limited lifespan, and its survival is a necessary prerequisite for fetal nutrition, even in unfavorable conditions. Placenta, not only supplies nutrients and oxygen to the fetus and removes waste products, but also functions as an endocrine organ throughout the pregnancy. It is a rapidly developing organ characterized by different types of villi formed throughout gestation.[1,2] The placenta accumulates and metabolizes maternal thyroxine (T4) and triiodothyronine (T3), and these maternal thyroid hormones may regulate fetal development via effects on the placenta.[3]

Thyroid disease in pregnancy results in conditions that affect both the mother and the fetus with potential important subsequent consequences for child development.[4] Hypothyroidism is traditionally defined as deficient thyroidal production of thyroid hormone. Hypothyroidism, usually evident as subclinical hypothyroidism, occurs in around 2.5% of otherwise normal pregnancies and, although relatively asymptomatic, is associated with pre-term delivery as well as an increased incidence of abortion, obstetric complications, fetal abnormalities, pre-eclampsia, low birth weight, and fetal death, if untreated.[5-7] This is of importance because the spectrum of thyroid deficiency begins with subclinical hypothyroidism characterized by elevated serum thyrotropin (thyroid-stimulating hormone, TSH) concentration in the presence of a normal serum free thyroxine level.[8,9] Maternal hypothyroidism during pregnancy disturbs rat and human fetal brain development resulting in neurological deficits in offspring.[10-12] Recently, Haddow et al. reported that untreated hypothyroidism during pregnancy may cause a significant decrease in the IQ of children.[13]

Programmed cell death or apoptosis is a fundamental biological phenomenon that occurs under a wide variety of physiological and pathological situations. The process of apoptosis was described by Kerr et al.[14] Apoptosis is characterized by chromatin condensation, membrane blebbing, nuclear breakdown and formation of apoptotic bodies.[15] Recently, several studies have indicated that apoptosis plays a role in the normal development, remodeling, and aging of the placenta.[16-20]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. The anti-apoptotic bcl-2 protein and pro-apoptatic bax protein play key roles in apoptosis. Caspase-3 is involved in the development of apoptotic cell death.[21-26]An abnormal level of apoptosis has also been correlated with a large variety of gestational pathologies such as in the placentas of abortions, ectopic pregnancy, intrauterine growth retardation, post-term pregnancy, pre-eclampsia and maternal hypertension syndrome.[17,27-29] Apoptosis has occasionally been observed in histological sections of normal thyroid.[30] However, apoptotic cell death is abnormally accelerated during the pathological phases leading to clinical hypothyroidism.[31]

The aim of this study was to demonstrate the structure of placenta histologically in normal pregnancy and maternal hypothyroidism, and to address the mechanism of apoptosis with immunohistochemistry and TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay.

MATERIAL AND METHODS

In this study, tissue samples were taken from archives and identitity of patients were hidden. In prospective studies that are not drug or experimental studies, Dokuz Eylül University ethics commitee approval is not needed, as in our study. Seven mothers with uncomplicated normal pregnancies and eight mothers with hypothyroidism aged between 26-40 years were selected from patients admitted to Dokuz Eylül University Hospital. All placentas were obtained after delivery at term and were sent to the Department of Pathology for histological examination.

Specimens were fixed in 10% formalin solution at room temperature for 24-48 hours and then a routine parafin procedure was performed. Sections (5 mm thick) were cut and prepared for both histochemical and indirect immunohistochemical stainings. Masson's trichrome stain was applied for

morphometric examinations.The slides were examined under a light microscope (Olympus BH-2, Tokyo, Japan) and the findings in both groups were compared.

IMMUNOHISTOCHEMICAL ASSESSMENT

For immunohistochemical evaluation, the avidin–biotin peroxidase system was used. For the immunohistochemistry procedure, Caspase-3, and Bcl-2 and Bax were employed. 5 μm thick sections were cut by microtome and were incubated at 60˚C overnight then dewaxed in xylene for 30 min. After rehydrating through a decreasing series of ethanol, sections were washed in distilled water for 10 min. They were then treated with 10 mM citrate buffer (AP-9003125, Labvision, Fremont, CA) at 95˚C for five minutes to unmask antigens by heat treatment. Slides were cooled in buffer for 20 minutes. Then slides were washed in deionized water three times for two minutes. Sections were delineated using a Dako pen (S2002, Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity.They were then incubated with normal serum blocking solution for 30 minutes. Sections were then incubated in a humid chamber overnight at 4˚C with antibodies to: Caspase-3 (MS-1121, Neomarkers, Fremont CA)(1/100, Mouse Monoclonal), Bcl-2 (MS-123, Neomarkers, Fremont CA) (1/100, Mouse Monoclonal) and Bax (MS-711, Neomarkers, Fremont CA) (1/100, Mouse Monoclonal). After this, they were washed three times for 5 min each with PBS, followed by incubation with biotinylated IgG and then with streptavidin-peroxidase conjugate (859042, Zymed, Fremont, CA). After washing, three times for 5 min with PBS, sections were incubated with DAB substrate containing diaminobenzidine (002020, Zymed, Fremont, CA) for 5 min to detect immunoreactivity and then with Mayer's hematoxylin. Sections were covered with mounting medium and were analyzed on a bright-field light microscope (Model BH-2, Olympus, Tokyo, Japan). Control samples were processed in an identical manner, just lacking the primary antibody step.

All sections were scored in a semi-quantitative fashion which took into consideration both the intensity and percentage of cells staining at each intensity. For each slide, a minimum of 5 fields was examined and assigned for severity of changes using scores of none (-), mild (+), moderate (++), and strong (+++).[32-34] For quantitative measurement of scoring of areas were randomly selected in these different areas and they were calculated. To maintain consistency of scoring, each section was graded by two person blind to treatments and the average was taken.

DETECTION OF THE APOPTOTIC CELL DEATH IN SITU USING THE TUNEL METHOD

TUNEL staining was mainly used for the identification or confirmation of apoptosis within the cells of the placenta. Apoptotic cell death was evaluated by in situ TUNEL kit (Cat. No:11 684 817 910 Roche, Germany) used for apoptotic cell detection. Briefly, serial 5μm thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and microwave-pretreated in trypsin solution at 37°C for 10 min (Roche, 10 109 819 001). After washing in phosphate-buffered saline (PBS), the specimens were incubated with fluorescein-labeled deoxy-UTP and TdT at 37°C for 60 min. Then, converter POD solution was applied to the slides. Sections were stained with DAB, counterstained with hematoxylin and mounted with Entellan. The percentage of TUNEL-positive cells was determined by counting the positive cells from 10 random fields in each group. The apoptotic index was defined as the number of apoptotic TUNEL-positive cells of placenta.

MEASUREMENT OF STROMAL FIBROSIS, MEAN THICKNESS OF BLOOD VESSELS AND NUMBER OF SYNCYTIAL KNOTS

All sections were stained with hematoxylin and eosin for mean thickness of blood vessels and the number of syncytial knots. Masson's trichrome stain was used for stromal fibrosis. The images were taken randomly for evaluating the thickness of blood vessels, the number of syncytial knots and stromal fibrosis. The images were taken by using a computer assisted video camera (JVC TK-890E, Japan) attached to a microscope (Olympus BH-2 Tokyo, Japan). The digital images were processed by an IBM-compatible

personal computer with an image analysis computer software (UTHSCA Image Tool for Windows version 3.0 software). In the digital images, the counting frame was placed randomly five times by the image analyzer computer program and the number of areas were counted and the average was taken. Mean thickness of blood vessels and the number of syncytial knots were calculated by UTHSCA Image Tool. In the counting process of the number of syncytial knots, 262417,5 μm^2 digital area owning counting frame was used. Measurements of stromal fibrosis areas were also determined using another digital image analysis software: Reconstruct version 1.0.8.2 (1996-2006 John C Fiala).

Statistical Analysis

All values were expressed as the mean ± standard deviation. The Mann–Whitney U-test was used to compare staining intensity values between groups ($P< 0.05$). All statistical procedures were performed by SPSS software for Windows, Version 11.0 (SPSS, Chicago, IL, USA). A value of $P< 0.05$ was considered significant.

RESULTS

Table 1 summarizes the clinical characteristics and statistical analysis of the patients. There were no significant differences for placental weight, maternal age and gestational age between the two groups. Immunohistochemical analyses of the Caspase-3, Bcl-2 and Bax in the human placenta are shown in Table 2.

Positive immunostaining for Caspase-3 there was moderately in the syncytiotrophoblast cells while it was mild in cytotrophoblast cells of the control group (Figure 1). However, mesenchymal cells were not stained for Caspase-3 in the control group. Caspase-3 immunostaining was strong in the syncytiotrophoblast, cytotrophoblast and mesenchymal cells in the hypothyroid group (Table 2) (Figure 1).

Immunostaining of Bcl-2 was moderate in the syncytiotrophoblast, cytotrophoblast and mesenchymal cells in the control group (Table 2) (Figure 2). In the hypothyroid group, Bcl-2 immunostaining was negative in the cytotrophoblast cells, mild in syncytiotrophoblast cells and focal and mild in the mesenchymal cells (Table 2) (Figure 2).

In the control group, Bax immunostaining was negative in the cytotrophoblast and mesenchymal cells, while immunostaining was mild in the syncytiotrophoblast cells (Table 2; Figure 3). In the hypothyroid group, Bax immunostaining was moderate in the syncytiotrophoblast and cytotrophoblasts; while it was mild in the mesenchymal cells (Table 2; Figure 3).

The difference between the mean number of syncytial knot formations was significant between

TABLE 1: Statistical analysis of clinical characteristics.

	Control group	Hypothyroid group	Statistical significance (p< 0.05)
Mean placental weight	581.4 ± 75.0	587.5 ± 105.0	Not significant
Mean maternal age	30.8 ± 1.8	31.0 ± 6.0	Not significant
Mean gestational age/day	249.7 ± 11.6	259.5 ± 15.0	Not significant

Data are shown as means and standard deviations.

TABLE 2: Distribution of Bcl-2, Bax, Caspase-3 and TUNEL in placenta.

	Control Group			Hypothyroid Group		
	Cytotrophoblast Cell	Syncytiotrophoblast Cell	Mesenchymal Cell	Cytotrophoblast Cell	Syncytiotrophoblast Cell	Mesenchymal Cell
Bcl-2	++	++	++	-	+	+/-
Bax	-	+	-	++	++	+
Caspase-3	+	++	-	+++	+++	+++

The intensity of immunostaining was graded as +++: Strong staining; ++: moderate staining; +:mild staining; -:none.

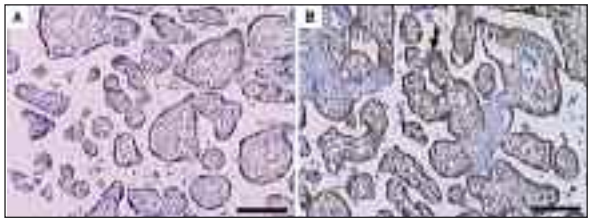

FIGURE 1: Immunohistochemical staining of Caspase-3 antigen in sections of placenta **(A)** Control group placenta **(B)** Hypothyroid group placenta. Caspase-3 positive immunostaining are seen(arrow) The scale bar in figure (A, B) represent, 100 µm.

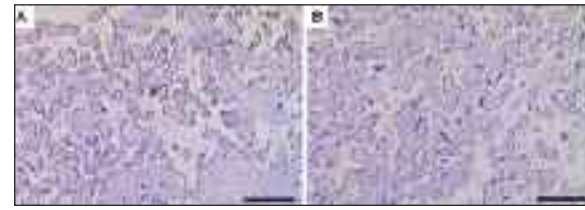

FIGURE 2: Immunohistochemical staining of Bcl-2 antigen in sections of placenta **(A)** Control group placenta **(B)** Hypothyroid group placenta. The scale bar in figure (A, B) represents 200 µm.

the groups (p< 0.05). Syncytial knots were significantly lower in the control group than in the hypothyroid group (Table 3). Mean thickness of medium size blood vessels was significantly lower in the hypothyroid group than in the control group (p< 0.05) (Table 3). Mean areas of stromal fibrosis in the hypothyroid group were higher than in the control group (p< 0.05) (Table 3; Figure 4).

The number of TUNEL positive cells in the hypothyroidic placentas were statistical significantly more than those in the control group (Figure 5).

FIGURE 3: Immunohistochemical staining of Bax antigen in sections of placenta **(A)** Control group placenta **(B)** Hypothyroid group placenta. Bax positive immunostaining are seen(arrow). The scale bar in figure **(A, B)** represent, 100 µm.

DISCUSSION

The fetus provides its own hormonal needs from the mother until its endocrine organs develope and function. One group of these hormones are the hormones of the thyroid gland. There are several studies which show that the isoform of human chorionic gonadotropin (HCG) displays a thyrotropic activity in the first trimester.[35,36] It is known that thyroid gland hormones in humans are important in the development of the central nerv-

TABLE 3: Mean number of syncytial knot formation, stromal fibrosis and thickness of blood vessels.

	Control group		Hypothyroid group		Statistical significant
	Mean ± SD	Median	Mean ± SD	Median	p< 0.05
Mean number of syncytial knot formation (*)	3.6 ± 1.0	3.0	8.8 ± 1.7	8.8	Significant (p= 0.010)
Mean number of areas of stromal fibrosis (Masson trichrome stain)(µm2)	167540 ± 88018	139302.6	234432 ± 61731	220501.6	Significant (p= 0.016)
Mean thickness of blood vessels (µm)	50.2±18.1	41.6	19.8±2.4	20.9	Significant (p= 0.020)

Data were shown as means and standard deviations.

* In the counting process of the number of syncytial knots, 262417,5 µm2 digital area owning counting frame was used.

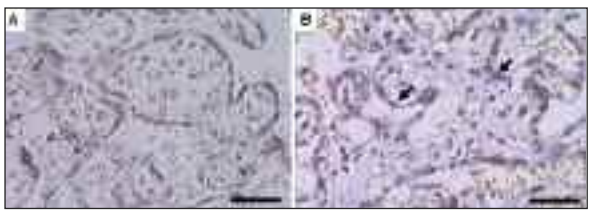

FIGURE 4: TUNEL Staining in sections of placenta. **(A)** Control group placenta **(B)** Hypothyroid group placenta. TUNEL positive cells are seen (arrow). The scale bar in figure **(A,B)**represents,50µm.

FIGURE 5: Masson Trichrom Staining in sections of placenta. **(A)** Control group placenta **(B)** Hypothyroid group placenta. Stromal fibrosis areas are seen. The scale bar in figure **(A, B)** represent, 200 µm.

ous system and essential for the normal development of many tissues. Therefore, the existence of fetal tissues and deiionidase, and levels of maternal plasma thyroid hormones within normal limits are essential for the healthy development of the fetus.[7,37-39] The clinical symptoms of the maternal hypothyroidism and insufficient secretion of maternal thyroid hormone cause problems in fetal development. There is increased risk of spontaneous abortion, or a decline in cognitive function in the babies who reach term, as seen in retardation in neurophysiological and psychomotor development, often with insufficiency in speaking and hearing.[40,41]

Although, there were no significant differences in placental weight, maternal age and gestational age between the two groups; the stromal and villous histopathological changes in the placenta like stromal fibrosis, medial coat proliferation of medium sized blood vessels and hyalinized areas were significant in the hyopthyroid group be the cause or effect of hypothyroidism. These findings correlate with findings of others who investigated apoptosis in growth retardation as a consequence of complicated pregnancies.[42-44]

As, in hypertensive mothers' placentas, the hypothyroid placentas also show a significant increase in syncytial knot formation. A significant increase in the syncytial knot formation in the placental villi may indicate a disturbance in the hormonal factors which may probably lead to altered morphology of the placenta resulting in pregnancy induced maternal hypothyroidism. These findings correlate with findings of others.[44,45]

Apoptosis is a programmed cell death in physiological and pathological conditions.[13] Recent studies have described apoptosis in both types of trophoblasts and have shown the importance of an apoptotic cascade for normal trophoblast function.[46]

Proteins related to regulatory pathways of apoptosis and cell death have been proposed in villous and extravillous trophoblasts of normal and complicated pregnancies.[18]

Abnormal apoptosis has been shown to be related to numerous gestational pathologies such as: placental abortus, ectopic pregnancy, intrauterine growth retardation, post-term pregnancy, pre-eclampsia and maternal hypertension syndrome.[47]

Moderate staining was observed after TUNEL staining in cytotrophoblast, syncytiotrophoblast and mesenchymal cells in term placentas of the control group where as strong staining was observed in all three cell types in hypothyroid placenta. These findings were consistent with other reports on placental apoptosis in growth retardation.[16, 48]

There were morphometric changes in placentas of hypothyroid mothers; The response to apoptotic and anti-apoptotic markers for Caspase 3 in cytotrophoblast cells was shown to be mild, syncytiotrophoblasts had moderate and mesenchymal cells had negative reaction in the control group. In the hypothyroid group, all three cell types showed a strong response.

Application of bcl-2 showed a moderate response in all three cell types in the control group, however there was negative response in cytotrophoblasts, mild response in syncytiotrophoblasts and mild focal response in mesenchymal cells in hypothyroidic placentas. These findings agreed with those of Aban et al. who researched complicated pregnancies.[26]

Bax immunoreactivity was negative in cytotrophoblasts and mesenchymal cells and was mild in syncytiotrophoblasts in the control group; whereas moderate response was observed cytotrophoblasts and syncytiotrophoblasts and mild response in mesenchymal cells in hypothyroid placentas. These findings correlate with findings of Cobellis et al. who investigated apoptosis in growth retardation as a consequence of pre-eclampsia.[49]

In spite of the histopathological and apoptotic symptoms, the restriction of intrauterine growth is not observed and normal birth might occur. This may result from sufficient thyrotropic activity by HCG in the first trimester. According to the level of the insufficiency of the activity of HCG, there might be aberrations such as deficiency in neuro-

physiologic and psychomotor development, insufficiency in speech and hearing and decrease in cognitive functions clinically.

In conclusion, we believe that serious permanent fetal growth retardation might occur in cases of maternal hypothyroidism with high incidence of apoptotic marker response and histopathological aberrations in the placenta.

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