ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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THE EFFECTS OF IMMOBILIZATION STRESS ON PLACENTA AND FETUS IN PREGNANT MICE

FARE GEBELİK DÖNEMİNDE HAREKETSİZLİK STRESİNİN PLASENTA VE YAVRUYA ETKİLERİ

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Öz

Amaç
Stres gebelik sürecinde anne ve fetüs sağlığını olumsuz etkilemektedir. Çalışmamızda kronik hareketsizlik stresinin plasenta ve fetüs gelişimi üzerine etkilerini araştırmayı hedefledik.

Gereç ve Yöntem
Balb/c suşu dişi fareler (20-30gr), 2dişi-1erkek olacak şekilde katıma alındı. Kontrol grubundaki (n=6) gebe farelere herhangi bir uygulama yapılmazken, stres grubundaki (n=6) gebe farelere gebeliğin 6.gününden 18.gününe kadar günde 3 defa 45dakikalık kronik hareketsizlik stresine maruz bırakıldı. Gebeliğinin 18. Gününde plasenta ve fetüsler anestezi altında sezaryen ile alındı.

Bulgular
Prenatal stres, trofoblastik dev hücreler, glikojen içeren hücreler ve labirent trofoblastik hücreler dahil olmak üzere birçok plasental hücrede apoptozu önemli ölçüde arttırdı ve intrauterin büyüme geriliğine sebep oldu. Stres süperoksit dismutaz ve glutatyon seviyelerini azalttı. Fetüsün gelişimini değerlendirmek için, Alizarin Red S boyaması ile fetüsün kemikleşme merkezleri değerlendirildi.

Sonuç
Gebelik sürecindeki stres, apoptozu tetikleyerek, labirent bölgesi küçüldü ve plasenta yetmezliğine sebep oldu, ayrıca kollajen seviyelerini arttırarak fetüs gelişimini olumsuz yönde etkileyerek intrauterin büyüme geriliği patogenezinde katkısı olduğunu gözlemledik.
Keywords: Fetus; hareketsizlik stresi; plasenta.

Abstract

Objective
Stress can affect negatively mother and fetuses during pregnancy. We aimed to investigate the effects of chronic immobilization stress on placental maturation and fetal development.

Materials And Methods
Balb/c virgin female mice (20-30 g) were mated with male mice in a 2 to 1 female to male ratio. Pregnant mice in control group (n=6) were left undisturbed, whereas pregnant mice in the stress group (n=6) were exposed to 45 min chronic immobilization stress for three times/day starting from gestational day 6 till 18. Fetuses and placentas were removed from dams on the gestational day 18 under anesthesia.

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Results
The prenatal stress significantly increased apoptosis in several placental cells including trophoblastic giant cells, glycogen cells and labyrinth trophoblastic cells and resulted in intrauterine growth restriction. The stress caused a decreased superoxide dismutase and glutathione levels. Alizarin Red S staining shows the ossification center of the fetuses to see developmental abnormality.

Conclusion
Gestational stress causes placental dysfunctions by triggering apoptosis, reducing the labyrinth zone as well as increasing collagen levels, which may impair fetal development that may contribute to pathogenesis of intrauterine growth restriction.

Keywords: Fetus; immobilization stress; placenta.

Introduction

The placenta is the most important role infetus development during pregnancy. If the placenta fails to function properly, fetal growth retardation or fetal death occurs. The mature placenta contains three layers; the labyrinth, the junctional zone (spongiotrophoblast), and the maternal decidua (1). The labyrinth zone is where maternal-fetal exchange occurs, consisting of syncitiotrophoblasts, chorionic trophoblasts, stroma, and blood vessels (2).

Stress can occur any time throughout the life and a useful experience that motivates an organism to overcome the pressure. Especially, if stress becomes chronic it may be injurious and destructive. The hypothalamus coordinates the stress as well as the metabolic responses. Many studies show the association of anxiety and depression with altered hypothalamus-pituitary-adrenal (HPA) axis. HPA system adapts the individual to the effect of stress. However, if the stress factor is often repeated, causing the chronic central drive to neurons controlling the stress response (3). Chronic stress may cause many harmful effects and may adversely affect life (4). Many studies show that the stress, during the pregnancy can cause of many pathological problems for the maternal and fetal side.

Reactive oxygen species (free radicals) cause metabolic or chemical reactions, disrupting intracellular and extracellular balance. Thus, oxidative stress occurs. Malondialdehyde (MDA) is lipid peroxidation product and one of the most important indicators of oxidative stress. The distruption balance between oxidative stress and antioxidant capacity, damages organs or organ systems. Free radicals is formed by endogenous and exogenous stress and it causes many diseases such as atherosclerosis, cancer, neurodegenerative diseases, drug-associated toxicity and harm placental development. Glutathione (GSH), antioxidant vitamins (vitamin E, vitamin C) and antioxidant enzymes (superoxide dismutase (SOD)) play an significant role in protecting cells against oxidative damage (5).

Apoptosis, that keep normal tissue function, is a programmed cell death and it is active process. Apoptosis in normal tissue turnover is called “programmed cell death”. However, sometimes apoptosis may be induced by the influence of cytokines or growth factors through external factors. Apoptosis by cause of stimuli has two known pathways: intrinsic-mitochondrial pathway, extrinsic-death receptor-mediated pathway or exogenous stimuli such as cytokines. Main stimulants of apoptosis are caspases which are a family of cysteine proteases separates a large number of vital cellular proteins to effect apoptosis cascade (6). Flice-like inhibitory proteins (FLIPs), inhibitors of apoptosis (IAP), and anti–apoptotic Bcl-2 family members, are some endogenous inhibitors of caspase, thus precluiding further spread of the death signal. In normal pregnancy, apoptotic cells are shown in both parts of the placenta, maternal and fetal sites and, existence of these cells is correlated the stages of placental development like trophoblast invasion, spiral artery transformation, trophoblast differentiation and parturition. Also, apoptosis has also been shown to be involved in the development of maternal immune tolerance against paternal antigens (7,8). Trophoblast apoptosis is seen more often and the steps of regulation of this apoptosis can shed light on the pathophysiology of these diseases. By controlling the release of pro-apoptotic factors from mitochondria, family members of Bcl-2 can adjust the death signals directly or intrinsically. The Bcl-2 family consists of three functional groups and they have a different number of Bcl-2 homology domains. Syncytiotrophoblasts were found to express higher levels of Bcl-2 than sitotropoblasts during pregnancy (8-12).

Morphological changes of apoptosis have been observed in uncomplicated pregnancies in villous trophoblast of placenta, including nuclear condensation, membrane overflow and DNA fragmentation, suggests that apoptosis is associated with the normal tissue remodeling.

Stress can affect negatively mother and fetuses during pregnancy. The period of pregnancy and duration of applied stress is important. This study evaluates the effects of chronic immobilization stress (CIS) on the placenta morphologically, biochemically (oxidant and antioxidant balance), immunohistochemically (apoptosis markers) and fetus in mice.

Materials And Methods

Animals

Balb/c mice supplied by Department of Laboratory Animal Sciences (Izmir, Turkey). The animals were kept at room temperature (23 ± 2 ° C) and humidity (60%), 12 hours light / dark cycle, and in an ad libitum food and tap water diet during the experiments.

Animal Experiments

All experiments were performed between January 2013 and March 2013 in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Ethical Committee of the Dokuz Eylul University, School of Medicine (No; 79/2013).

Before starting the experiment, all animals were provided pregnancy with superovulation. Superovulation was achieved by 5IU FSH (Puregon, Organon) and then 5IU human chorionic gonadotropin (hCG) (Pregnyl, Organon), over a 48 hour period. Because pregnancy possibility with superovulation is higher than spontan cyclus (13). After the hCG injection, females were placed in cages with males and left to copulate and vaginal plug was evaluated the next day. Vaginal plug means mating and its presence defines the first day of pregnancy.

The pregnant mice were divided into 2 groups. Control group: Pregnant (n=6), protected group during pregnancy. Stress group: pregnant and stressed group (n=6). Stress group 6 days in the cages were kept stress-free. Then, between 6 and 18 days of gestation, subjected to CIS three times a day (for a duration of 45 minutes) between 09:00, 12:00 and 17:00 pm. We excluded preimplantation period (E0.5-E6.0) and included twelve days (E6.0-E18.0) for chronic stress model.

Female mice in both groups were caesarean under ether anesthesia on the 18th day of their pregnancies and placentae and pups were taken.

Chronic Immobilization Stress Model

Stress group 6 days in the cages were kept stress-free. Then, between 6 and 18 days of gestation, subjected to CIS three times a day (for a duration of 45 minutes) between 09:00, 12:00 and 17:00 pm. Animals were kept in ventilated conical flexible tubes (width 5.5 cm; base to top 2.5 cm; length 5.5, 6.5 or 7.5 cm depending on size of mouse) (14).

Histological, Morphological and Biochemical Evaluation

The placental materials to be used for histological evaluations were taken into 10% formaldehyde for 48 hours. Then by applying routine tissue processing procedure, the tissues were embedded in paraffin blocks.

From all tissue blocks sections of 5 µm were cut and stained with H&E (01562E&01602E, Surgipath, Bretton, Peter Borough, Cambridgeshire) hematoxylin solution for 5 min and eosin solution for 10 s for histological analyses.

The placental materials to be used for biochemical examinations were taken into cryovial by washing with PBS. Placental tissues were prepared homogenate for biochemical analyses (Superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA)). Plecantal tissues were washed with saline solution twice and homoganated with Tissue Lyser Machine. SOD, MDA, GSH were measured by HPLC spectrometer (GSH: cat no 354102-Meck, SOD: 574601-Merck, MDA:820756-Sigma Aldrich).

The numbers of total fetus, as well as live and dead fetuses, were recorded. Also, placental and fetal weights were measured. After examination of live fetuses for the external morphologic abnormalities, fetuses were fixed with 96% ethanol and stained with Alizarin Red S (A5533, Sigma), and examined for the skeletal malformations and developmental restrictions by staining ossification areas in the fetus.

Immunohistochemistry

After deparaffinization and rehydration, sections were then treated with 10 mM citrate buffer (Cat No. AP-9003-125 Labvision) for 5-minute sections were incubated in a solution of 3% H2O2 for 5 min to inhibit endogenous peroxidase activity. They were then incubated with normal serum blocking solution. Sections were then incubated in a humid chamber for 18 h at

+4ºC with anti-Caspase-3 (Cat No. AB3623-Milipore) /Bcl-2 (Cat No. AB182858-Abcam) /Bax antibody (Cat No. AB2915-Milipore) 1:100. Sections were then incubated with biotinylated IgG and then with streptavidin conjugated to horseradish peroxidase for 30 minutes each prepared according to kit instructions (Invitrogen-Plus Broad Spectrum 85-9043). Finally, the sections were stained with DAB, counter-stained with Mayer hematoxylin, and analyzed using a light microscope. Staining intensity of tissue sections was evaluated and graded (0, absence;1, weak; 2, moderate; 3, strong) in a blinded fashion by two examiners and assessed by using the HSCORE. The HSCORE was calculated using the fallowing equation: HSCORE=-Pi (i+1), where i is the staining intensity and Pi the percentage of stained placental cells at each level of intensity.

Statistical Analysis
Immunostaining HSCOREs for each antibody in placental tissue results were compared by a t-test. Biochemical results were analyzed with one-way analysis of variance (ANOVA) post hoc Bonferroni test. $p<0.05$ is accepted as statistically significant. Statistical calculations were performed using by Sigma Plot version.3.

Results

Body Weight Measurements
At the end of the experiment, the weights of the mothers were 42.19 g in the control group and 31.31 g in the stress group. There is a significant difference between two groups ($p< 0.05$) (Fig 1A). The weights of the fetuses obtained at the end of the experiment were 1.20 g in the control group and 0.77 g in the stress group. There is a significant difference between two groups ($p<0.05$) (Fig 1B). When placenta weights are measured, the control group mean is 0.17 g and stress group mean is 0.17 g. There is no significant difference between the two groups (Fig 1C).

Macroscopic Results
Pregnant mice were sacrificed at 18 days of gestational age. 47 fetuses were alive and 2 fetuses died from the control group and 31 were alive and 10 fetuses were died from stress group in for which total 90 fetuses were collected by cesarean section. There was no malformation of fetus such as abnormally of skull bones, skin, and vertebrae, thoracic, harelips. Whole fetuses were stained by the Alizarin Red S to determine for intrauterine growth retardation. Alizarin Red S staining shows ossification center of fetuses' skeleton (Fig 2). Some fetuses of stress group had early skeletal development (Fig 3).

Histological Results
In the histological evaluation, placental tissues in control and stress groups were morphologically normal (Fig 4).

In addition to the morphological analysis of H&E stained sections, the lengths of the different layers in the placenta were quantitatively measured using a Zeiss Imager A2 microscope and the Axiocam program and statistically analyzed. Labyrinth zone, 1474.81 µm in the control group, 1185.78 µm in the stress group (Fig 5A); junctional zone, 664.48 µm in the control group, 664.43 µm in the stress group (Fig 5B); while the decidua basalis was measured as 33.92 µm in the control group and 56.41 µm in the stress group (Fig 5C).

When the glycogen cell areas examined, there was no significant difference between the groups (Fig 6A). In addition, areas of the glycogen are measured using the Zeiss Imager A2 Axiocam program. The mean of the glycogen cell areas in the control group was 762.55 µm2, and the stress group was 997.00 µm2 (Fig 6A).

Histological analysis showed that increase in connective tissue areas in placentas of the stress group (550.13 µm2) compared to that of the controls (388.51 µm2) ($p<0.05$) (Fig 6B).

Immunohistological Results
The expression of Active-Caspase-3, Bax and Bcl-2 in paraffin embedding tissue sections from both groups were analyzed by immunohistochemistry staining.

Active Caspase-3 immunoreactivity was significantly higher in the stress group's placental trophoblastic cells and glycogen cells compared to that in the control group's placentas (Fig 7) ($p<0.05$). In addition, Bax immunoreactivity was increased in stress group's placental trophoblastic giant cells ($p<0.005$), fetal vessel endothelial cells ($p<0.001$) and labyrinth trophoblastic cells ($p<0.001$) compared to that in control group's placentas (Fig 8). However, Bcl-2 immunoreactivity was no significant difference between the groups (Fig 9).

Biochemical Parameters
Samples of placentas were cumulated for biochemical analyses. According to biochemical analyses, we found significantly decrease in SOD and GSH levels in the stress group ($p<0.05$) whereas a non-significant increase in MDA level was detected in the stress group compared to control group ($p>0.05$) (Fig 10).

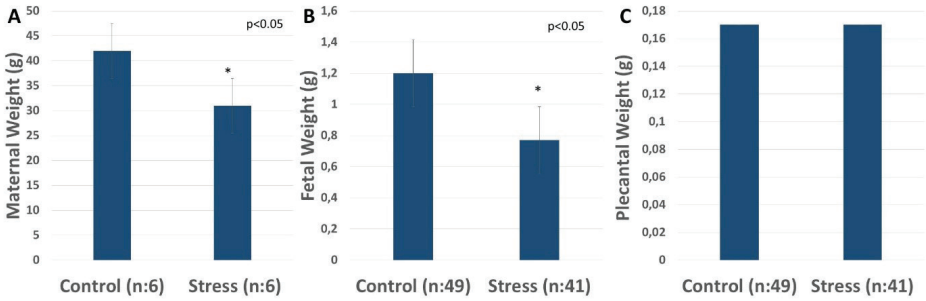

Figure 1
Chronic stress affects maternal and fetal weight. (A) Maternal weight, (B) fetal weight and (C) placental weight. The bars represent the mean ± S.E.M. (* p<0.05).

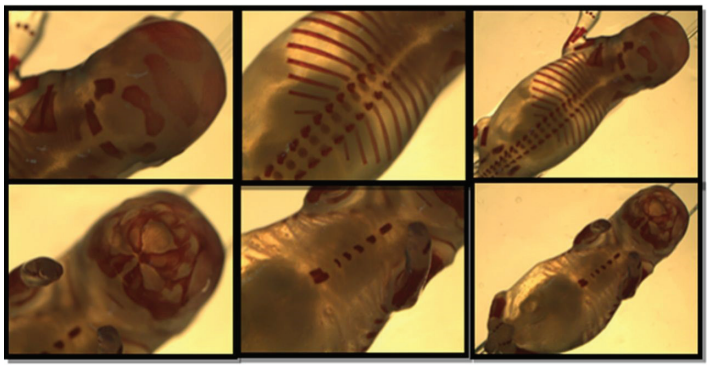

Figure 2
Images of control fetuses stained with Alizarin Red S. Red stained areas are ossification centers.

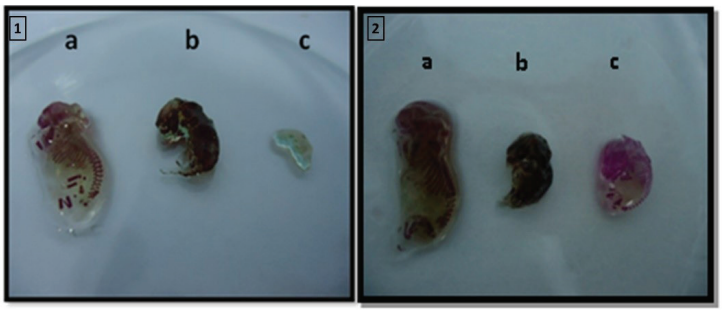

Figure 3
Images of chronic inactivity stress group fetuses stained with Alizarin Red S. Red stained areas are ossification centers. Figure 3.1 is three fetuses of a mother belonging to chronic inactivity stress group belong to different prenatal developmental stages. Figure 3.2 is three fetuses of another mother belonging to chronic inactivity stress group belong to different prenatal developmental stages.

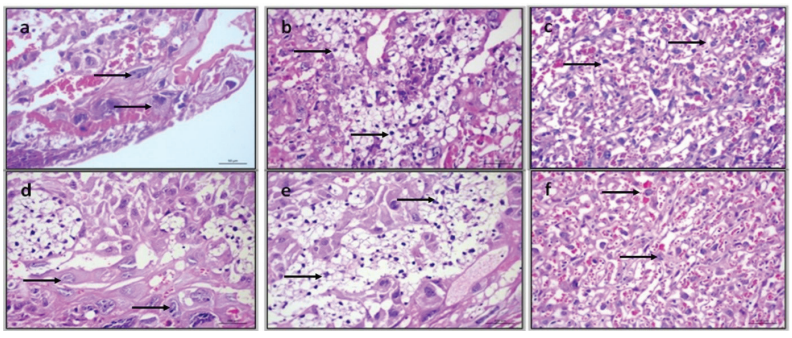

Figure 4
Placental areas of control and chronic inactivity stress group. Control group (a) trophoblastic giant cells, (b) glycogen cells, (c) spongio-trophoblastic cells (x400). Stress group (d) trophoblastic giant cells, (e) glycogen cells, (f) spongio-trophoblastic cells (x400). Scale bar:50 µm.

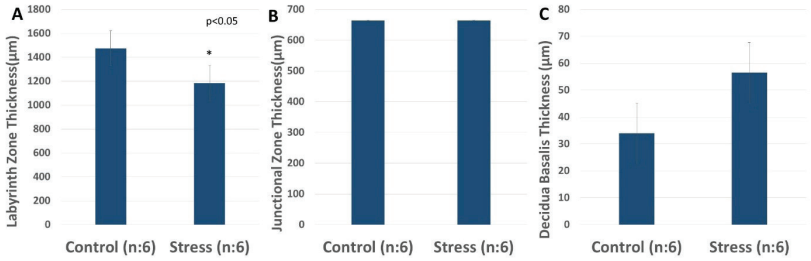

Figure 5
Placental zones measurements in H&E staining. (A) Labyrinth zone thickness, (B) junctional zone thickness, (C) decidua basalis thickness. The bars represent the mean ± S.E.M. (* p<0.05).

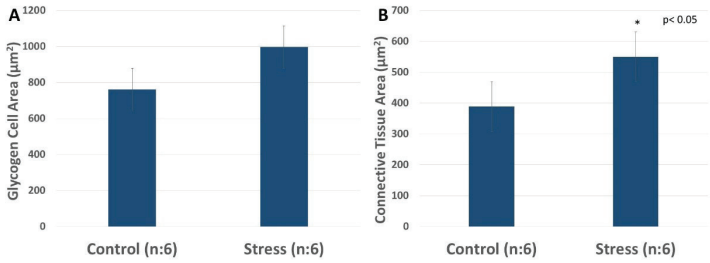

Figure 6
(A) Glycogen cell area, (B) Connective tissue area in placentas (* p<0.05). The bars represent the mean ± S.E.M.

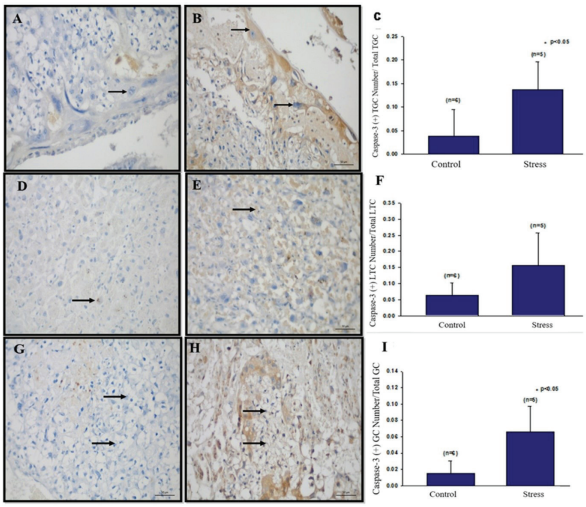

Figure 7
(A, B) The active Caspase-3 activity of trophoblastic giant cells, (C) the graph shows the ratio of the mean active aspase-3 (+) trophoblastic giant cells to the total trophoblastic giant cells in the control group and the stress group. (D, E) The active Caspase-3 activity of labyrinth trophoblastic cells, (F) the graph shows the ratio of the mean active caspase-3 (+) labyrinth trophoblastic cells to the total labyrinth trophoblastic cells in the control group and the stress group. (G, H) The active Caspase-3 activity of glycogen cells, (I) the graph shows the ratio of the mean active Caspase-3 (+) glycogen cells to the total glycogen cells in the control group and the stress group. (A, D, G) Control group, (B, E, H) stress group (TGC: Trophoblastic giant cell, LTC: Labyrinth trophoblastic cell, GC: Glycogen cell). The active Caspase-3 activity was calculated with HSCORE. The bars represent the mean ± S.E.M (* p<0.05). Scale bar:50 µm.

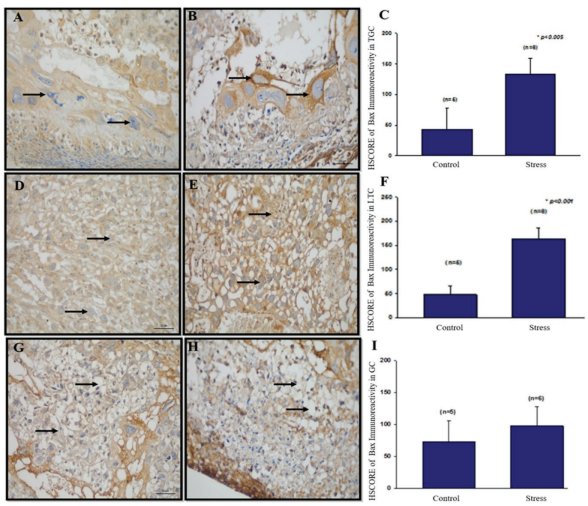

Figure 8
(A, B) Bax immunoreactivity of trophoblastic giant cells, (C) graph shows the semi-quantitative results of Bax immunoreactivity of the trophoblastic giant cells in the control and stress groups. (D, E) Bax immunoreactivity of labyrinth trophoblastic cells, (F) graph shows the semi-quantitative results of Bax immunoreactivity of labyrinth trophoblastic cells in the control and stress groups. (G, H) Bax immunoreactivity of glycogen cells, (I) graph shows the semi-quantitative results of Bax immunoreactivity of the glycogen cells in the control and stress groups. (A, D, G) Control group, (B, E, H) stress group (TGC: Trophoblastic giant cell, LTC: Labyrinth trophoblastic cell, GC: Glycogen cell). The bars represent the mean ± S.E.M (* p<0.005, p<0.001). Scale bar:50 µm.

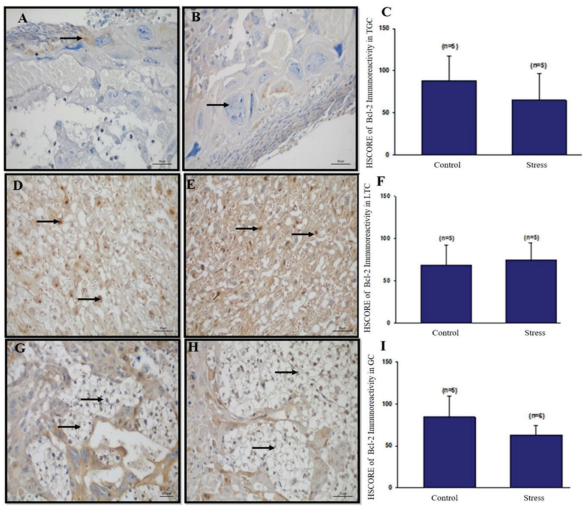

Figure 9
(A, B) Bcl-2 immunoreactivity of trophoblastic giant cells, (C) graph shows the semi-quantitative results of Bcl-2 immunoreactivity of the trophoblastic giant cells in the control and stress groups. (D, E) Bcl-2 immunoreactivity of labyrinth trophoblastic cells, (F) graph shows the semi-quantitative results of Bcl-2 immunoreactivity of labyrinth trophoblastic cells in the control and stress groups. (G, H) Bcl-2 immunoreactivity of glycogen cells, (I) graph shows the semi-quantitative results of Bcl-2 immunoreactivity of the glycogen cells in the control and stress groups. (A, D, G) Control group, (B, E, H) stress group (TGC: Trophoblastic giant cell, LTC: Labyrinth trophoblastic cell, GC: Glycogen cell). The bars represent the mean ± S.E.M. Scale bar:50 µm.

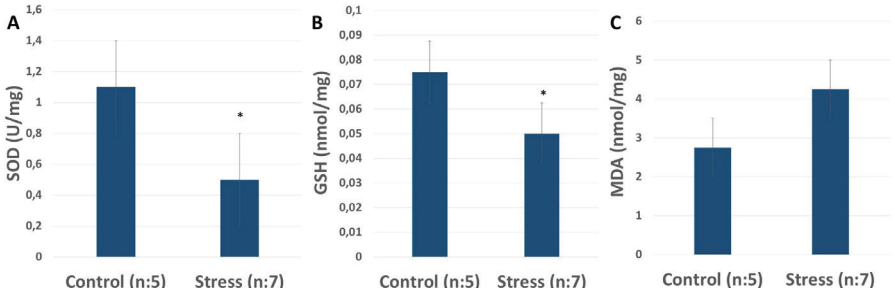

Figure 10
Biochemical results. (A) SOD levels, (B) GSH levels and (C) MDA levels. The bars represent the mean ± S.E.M (* p<0.05).

Discussion

Daily maternal weight gain is a result of the developing placenta and fetus and increasing maternal tissue (nutrients, extracellular liquid, blood volume, fat tissue and breasts) during pregnancy besides fetal growth (15). Our result supports that maternal stress cause decreased maternal body weights gain (16). In additionally, IUGR is clinically defined as a birth weight below the tenth centile for gestational age where the fetus does not meet its growth potential (17). Analyses of fetal weight showed that CIS decreases significantly fetal body weight, which may be associated with IUGR (18).

The placenta plays a critical role in modulating maternal-fetal resource allocation. It may be effect on fetal growth and fetus' health. Placenta' metabolically active tissue responds some chaos by regulating the fetal supply of nutrients and exchange of oxygen-carbon dioxide between fetus and maternal blood (19). Our results showed that there are no distinct differences between the two groups' placentas, which stained with hematoxylin, and eosin for histological examination.As a result, chronic stress don't affect morphology of placenta, but it doesn't mean that function has no effect.

Placental zones were same order from the maternal to the fetal surface these are; 1) The Decidua Basalis, 2) The Basal Zone and 3) The labyrinth zone besides placental cells were in right zone. The labyrinth zone contains fetal vessels. If the labyrinth zone is not appropriately vascularized with suitable patterning,branching and dilation, placental perfusion is impaired, resulting in poor oxygen and nutrient difusion (20). Our results showed that the labyrinth zone of stress group's placenta was significantly thinner. This result may explain etyology of low fetal weight.

We didn't find any studies that looked at Bax, Blc-2, or Caspase-3 immunoreactivity in literature reviews.

In this context, the effect of inactivity stress on the expression and activity of Bax, Blc-2 or Caspase-3 in placental cells was shown for the first time in our study.

Trophoblastic giant cells directly connect to the maternal tissue on the outermost part of the extraembryonic region and secrete cytokines, which are hormones specific to pregnancy (9). Labyrinth trophoblastic cells are located close to the maternal blood vessels and help transport in the feto-maternal circulation (10,11). The glycogen cells stock glycogen to supply the fetal energy need (12). As a result of our immunohistochemical analysis, we found that Caspase-3 immunoreactivity increased significantly in placental trophoblastic giant cells and glycogen cells in the stress group. The increase in Caspase-3 immunoreactivity is directly related to cell death. In this sense, increased apoptosis can be mentioned both in trophoblastic giant cells and in glycogen cells. This will lead to a reduction in placental functions of these cells due to a decrease in the number of trophoblastic giant cells and glycogen cells. These results support our thought that it may be one of the main causes leading to the decrease in fetal weights due to inactivity stress.

Bax is a pro-apoptotic protein that can induce cell apoptosis independently of the Caspase-3 pathway. The Bax pathway usually occurs via mitochondria (21). In our study, changes in Caspase-3 activity are observed in trophoblastic giant cells and in glycogen cells. The change of Bax level in labyrinth trophoblast cells suggests that placental cells go apoptosis from different pathways. In this sense, we can say that labyrinth trophoblast cells go into apoptosis due to the mitochondrial pathway. This apoptotic effect may occur as a secondary event due to the direct effect of immobilization stress due to the decreased apoptosis of the glycogen cells, as well as the reduction of placental glycogen and energy sources. Previous studies have also reported findings indicating increased oxidative stress (22). We may also show another reason for the stimulation of mitochondrial apoptosis in oxidative stress in placental cells caused by inactivation stress in our biochemical parameters.

Reactive oxidative species sometimes harm placental development. In our study, antioksidant factors (SOD, GSH) of stress group's placenta was significantly lower and MDA (oxidant factor) is no distinct differences between the two groups' placentas. We can say that unchanged MDA levels caused to decrease SOD and GSH levels. The result is increased oxdative stress.

OS (oxidative stress) can stimulate apoptosis via external or intrinsic signals and also block some key apoptotic regulators, such as proteins of the Bcl-2 family. Bcl-2 family proteins play a important role in the intrinsic pathway of apoptosis (23). And also, excessive OS is associated with the pathologies of spontaneous abortion, PE and IUGR. According to our immunohistochemistry results; we conclude that CIS increases oxidative stress and demonstrated that maternal stress induces the apoptosis of placental cells, causes histologic damages in placental tissue. These placental damages are likely to cause a partial negative effect during fetal development.

Conclusion

These results suggest that gestational stress causes placental dysfunctions by triggering apoptosis, reducing the labyrinth zone as well as increasing collagen levels, which may impair fetal development that may contribute to pathogenesis of IUGR.

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