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Evaluation of chondrogenesis and osteogenesis via Wnt/β-Catenin, S100 immunoexpression and histomorphometry in fetal rats following maternal uterine artery ligation

Maternal uterin arter ligasyon modeli uygulanan fetal sıçanlarda kondrogenez ve osteogenezin Wnt/β-Catenin, S100 immünoekspresyon ve histomorfometri ile değerlendirilmesi

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Abstract

Aim: The aim of this study is to investigate the effects of intrauterine growth retardation depending on maternal uterine artery ligation model, Wnt/β-catenin and S100 expression immunohistochemistry and histomorphometrically on growth plate and bone tissue of fetal rats.

Materials and Methods: Maternal rats were randomly divided into 3 groups (n=5). No surgery or anesthesia were applied in control group. Bilaterally the maternal uterine arteries were ligated on gestational day 18 in experimental group. Although all surgical procedures were performed in sham group, the uterine artery ligation were not made. Fetuses were taken on gestational day 20, thicknesses of growth plate and zones, trabecular number and thickness and cortical thickness were evaluated with the histomorphometrically in samples from left proximal tibia. The expressions of β-catenin and S100 immunohistochemically were evaluated in the growth plate.

Results: Thicknesses of growth plate ($p<0.01$), proliferation zone ($p<0.05$) and degeneration zone ($p<0.01$) were measured significantly thinner in experimental group than the others and thicknesses of hypertrophic zones were lesser than the control and sham group, but the results were not statistically significant ($p>0.05$). Also trabecular numbers were lower ($p<0.01$) and trabecular thickness were also thinner ($p<0.05$) in experimental group. Expression of β-catenin was declined and S100 expression was increased in experimental group.

Conclusion: We conclude that maternal uterine artery ligation, leads to shortness of growth plate and degenerated bone architecture because of Wnt/β-catenin signaling pathway.

Keywords: Intrauterine growth retardation, uterine artery ligation, growth plate, β-catenin, S100, histomorphometry.

Öz

Amaç: *Bu çalışmanın amacı, sıçanlarda maternal uterin arter ligasyonuna bağlı olarak gelişen intrauterin büyüme geriliği modelinde büyüme plağı ve kemik dokuda Wnt/β-catenin ve S100 ekspresyonlarının immünohistokimyasal ve histomorfometrik olarak değerlendirilmesidir.*

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Gereç ve Yöntem: *Gebe sıçanlar üç gruba ayrıldı (n=5). Kontrol grubuna cerrahi veya anestezi uygulanmadı. Deney grubunda, gebelik 18. gününde maternal uterin arterlere bilateral ligasyon yapıldı. Sham grubuna uterin arter ligasyonu dışındaki tüm cerrahi işlemler uygulandı. Fetuslar gebeliğin 20. gününde alındı, sol proksimal tibiadan büyüme plağı ve zonlarının kalınlıkları, trabeküler sayı ve kalınlıkları, kortikal kalınlıklar histomorfometrik olarak ölçüldü. Büyüme plağında β-catenin ve S100 ekspresyonları immunohistokimyasal olarak değerlendirildi.*

Bulgular: *Deney grubunda büyüme plağı (p<0,01), proliferasyon zonu (p<0,05) ve dejenerasyon zonun kalınlıkları (p<0,01) belirgin olarak daha ince bulundu. Hipertrofik zonların kalınlığı daha az olarak ölçüldü (p>0,05). Aynı zamanda daha düşük sayıda trabekülar yapı (p<0,01) ve daha ince trabeküler kalınlık (p<0,05) bulundu. Deney grubunda β-catenin ekspresyonu daha düşük iken S100 ekspresyonunda artış saptandı.*

Sonuç: *Bu çalışma, maternal uterin arter ligasyonuna bağlı fetal sıçan intrauterine büyüme geriliği modelinde, Wnt/β-catenin sinyal yolağındaki azalmaya bağlı olarak dejenere kemik yapısı ve büyüme plağında kısalmaya yol açtığını göstermektedir.*

Anahtar sözcükler: *İntrauterin büyüme geriliği, uterin arter ligasyonu, büyüme plağı, β-catenin, S100, histomorfometri.*

Introduction

Intrauterine growth is modulated by blood flow in which oxygen, nutritional and hormonal factors are delivered through mother to fetus. If this blood flow is restricted or slowed down, fetal development is affected resulting in intrauterine growth retardation (IUGR). Intrauterine growth retardation causes perinatal mortality and some serious disorders such as heart, brain and bone defects (1-5).

Development and growth of long bones, which is a complicated process, consists of stem cell proliferation, differentiation, synthesis and remodeling of bone extracellular matrix and growth plate (6). Growth plates generate a model for development and grow into long bones via endochondral ossification. Mesenchymal stem cells (MSCs) differentiate into cartilage cells and these cells synthesize and secrete type II collagen fibers and extracellular matrix, and then this cartilaginous model differentiates into bone (1, 6-10). Growth plate consists of four histological layers consisting of reservation, proliferation, hypertrophic and degeneration zones. The cartilage cells of reservation zone synthesize cartilage matrix and function as stem-like cells. Proliferating chondrocytes are located in the proliferative zone. Degenerative zone is the last zone of the growth plate. Blood vessels invade this zone and bring osteoprogenitor cells which generate into bone tissue (11-13).

Developmental processes of cartilage and bone are regulated by Wnt proteins. Wnt proteins, which are part of canonical and non-canonical signaling pathways, consist of 19 members. Wnt/β-catenin constitutes canonical pathway. This pathway affects proliferation and differentiation of MSCs into cartilage in endochondral ossification process at early stages and bone cells metabolism (9, 14-18). S100 proteins play an essential role for cell survival, differentiation, growth and mobility. Moreover, these proteins affect cellular surface receptors involved in signal transduction in various cell types such as Schwann cells, glial cells and cartilage cells. S100 proteins are known to effective in cartilage matrix synthesis and repair (19-21). It is important to determine the amount of S100 proteins in endochondral ossification in relation to synthesis of collagen II in chondrocytes (20).

We investigate the effects of intrauterine growth retardation depending on maternal uterine artery ligation model, Wnt/β-catenin and S100 expression immunohistochemistry and histomorphometrically on growth plate and bone tissue of fetal rats.

Materials and Methods

Experimental and animal design

Total of fifteen pregnant rats, were obtained from the local Animal Ethics Committee at Ege University School of Medicine, were divided randomly into three groups (n=5) and rats were individually placed into the cages. The rats were acclimated to the local vivarium conditions (23-24˚C and 12/12 light-dark cycle) and fed commercial natural diet for one month. Vaginal smear was collected every morning at 09.00 o'clock to determined estrous cycle days. The female rats were mated with male rats during one night. Vaginal plaques were checked in the next morning. Rats with positive vaginal plaques (gestational day (GD) 0) were divided into three

groups next morning. All surgical procedures were done under sterile conditions and the rats were anesthetized using xylazine (6 mg/kg) and ketamine (30 mg/kg) intraperitoneally (2, 3). Experimental group received bilateral uterine artery ligation near the cervical end of the artery cascade on GD 18. Loss of pulsation in the artery was detected, bleeding control was performed and abdominal layers were closed in experimental group. Sham group received all surgical procedures except uterine artery ligation on GD 18. Control group received no surgical treatment (2, 3). All fetuses were delivered by caesarean section on GD 20. Left proximal tibias were dissected and fixed. After the 3 days decalcification in 10% formic acid, routine tissue processing was performed on all tissues in. Tissue sections of 5-µm were stained with Hematoxylin-Eosin (H&E), Safranin O and Masson's Trichrome for evaluation of growth plate and trabecular structure histomorphometrically. Expressions of β-catenin and S100 were determined via immunohistochemistry (22, 23).

Histomorphometrical analyses

In samples from left proximal tibia, thicknesses of growth plate, thickness of reserve, proliferative, hypertrophic and degenerative zones, trabecular number and thickness, and cortical thickness were evaluated with histomorphometry analysis. Histomorphometry was performed via Olympus BX51 (bright-field microscope, America Inc., USA). Briefly, the measurement of trabecular and cortical width was done directly at 5 different points. A minimum of 5 serial sections in each rats and all area in each section were quantified at a magnification of X40 and X100 in each section after processing by a semiautomatic digitizer from The University of Texas Health Science Center at San Antonio (UTHSCSA Image Tool for Windows Version 1.28) (5, 12, 23, 24, 25, 26).

Immunohistochemical analyses

Tissue sections of 5 µm were taken from paraffin blocks. The tissue blocks were chosen carefully after histological assessment of sections stained with hematoxylin and eosin (hematoxylin acc. to Gill III, Cat. no. 1.05174; eosin solution 0.5% alcoholic, Cat. no. 1.02439, Merck, Darmstadt, Germany). For immunohistochemical staining, sections were incubated overnight at 58˚C and then immersed in xylene and rehydrated through a series of ethanol solutions. Sections were washed with both distilled water and phosphate-buffered saline solution (PBS, P4417; Sigma-Aldrich, St Louis, MO) for 10 minutes and then treated with citrate buffer (ph:7,6) for 5 minutes at 90W and for 15 minutes at 360W in microwave oven. Following washing with PBS, sections were delineated with a Dako Pap pen (S2002, Dako, Glostrup, Denmark) and incubated in a solution of 3% hydrogen peroxide for 5 minutes to inhibit endogenous peroxidase activity. After washing in PBS, sections were incubated with nonimmune serum for 1 hour and then incubated with primary antibodies: β- catenin (1/100, Dako, Denmark), S100 (1/100, Dako, Denmark) overnight at 4˚C in a refrigerator. Sections were washed 3 times for 5 minutes each with PBS, followed by incubation with biotinylated secondary antibody and then with streptavidin-conjugated to horseradish peroxidase in PBS for 30 minutes each (Histostain-Plus Kit (HRP- 1000), Invitrogen). After washing 3 times with PBS, sections were incubated with DAB (liquid DAB-Plus substrate Kit, Invitrogen) for 10 minutes for immunostaining. After washing with distilled water, sections were counterstained with Mayer's hematoxylin (MHS1, Sigma-Aldrich, Deutschland) and washed with distilled water. All slides were evaluated by conventional light microscopy (Olympus BX51, Olympus America Inc., USA). The presence of a brown precipitate indicated positive reaction for primary antibodies. Expressions of β-catenin and S100 were scored weak (+1), moderate (+2) or strong (+3) (27).

Statistical analyses

Multivariate analysis of variation (MANOVA) tests were used to determine differences in various measurements among different groups. $p<0.05$ was considered significant.

Results

Histomorphometrical analyses

Thickness of growth plate and growth plate zones: Thickness of growth plates was found to be significantly reduced in experimental group (1609.61±56.09) than in the controls (2127.14±79.89) and in Sham group (2163.97±6966) ($p<0.01$ for each). When the thicknesses of the zones in the growth plate are measured, thickness of the proliferation zone (910.02±96.89) and degeneration zone (186.28±71.21) were found to be reduced in experimental group than in controls (894.76±99.68; 428.08±37.85 respectively) ($p<0.01$ for each). Thickness of hypertrophic zones in experimental group (602.68±62.12) were also reduced compared to those in control (705.41±95.47) and sham group (679.27±96.24), but the differences were not statistically significant ($p>0.05$ for each) (Figure-1a-c, Figure-2a-c, Table-1).

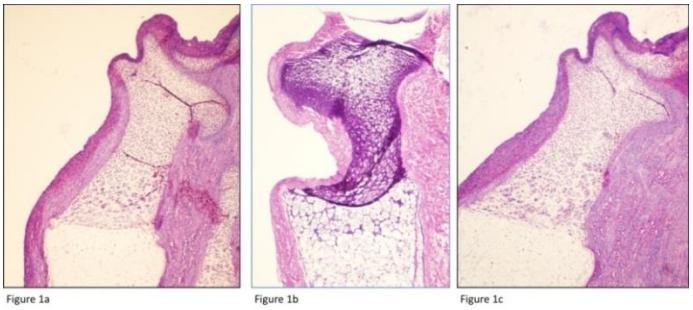

Figure-1. Photomicrographs of epiphyseal growth plate Hematoxylin-eosin X40, **a.** Control group. **b.** Experimental group. **c.** Sham group.

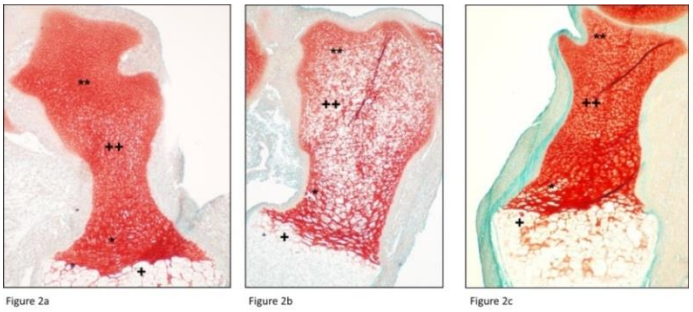

Figure-2. Photomicrographs of epiphyseal growth plate Safranin O X40 **a.** Control group. **b.** Experimental group. **c.** Sham group. **Reserve zone, $^{++}$Proliferative zone, *Hypertrophic zone, $^{+}$Degenerative zone.

Table-1. Thickness of epiphyseal growth plate zones mean±SD, a: p<0.05 when compared with control group, aa: p<0.01 when compared with control group, b: p<0.05 when compared with sham group, bb: p<0.05 when compared with sham group. All thickness values are expressed in pixels (1 pixel = 0.36 µm).

Thickness of epiphyseal growth plate zones (pixel) mean±sd	Growth plate zone	Rezervation zone	Proliferation zone	Hypertrophic zone	Degeneration zone
Control group	2127.14±79.89	98.89±28.25	894.76±99.68	705.41±95.47	428.08±37.85
Sham group	2163.97±69.66	106.14±30.47	910.02±96.89	679.27±96.24	468.54±42.9
Experimental group	1609.61±56.09	76.57±16.8	910.02±96.89	602.68±62.12	186.28±71.21

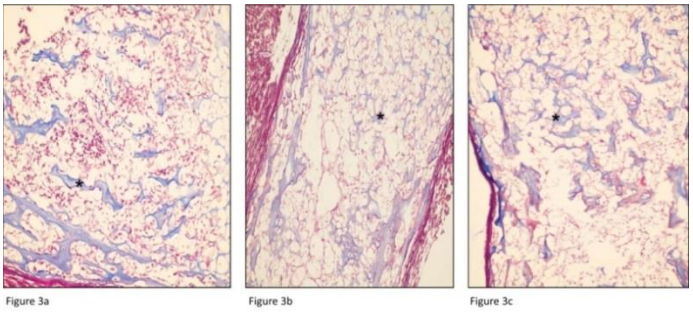

Figure-3. Representative photomicrographs of trabecular area Masson's Trichrome X100 **a.** Control group. **b.** Experimental group. **c.** Sham group. *Trabecula.

Table-2. Measurement of trabecular number, thickness and cortical thickness mean±SD, a: p<0.05 when compared with control group, aa: p<0.01 when compared with control group, b: p<0.05 when compared with sham group, bb: p<0.05 when compared with sham group. All thickness values are expressed in pixels (1 pixel = 0.36 µm).

mean±sd	Trabecular number	Trabecular thickness	Cortical thickness
Control group	37±5.05	38.87±10.92	36.07±8.43
Sham group	24.5±2.12	30.41±8.43	21.64±7.51
Experimental group	16.67±3.79	16.56±6.27	17.46±3.52

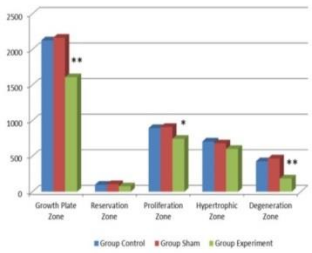

Figure 4a

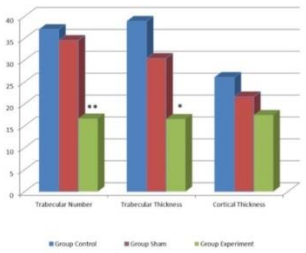

Figure 4b

Figure-4. Histomorphometrical analyses **a.** Thickness of epiphyseal growth plate zones. **b.** Measurement of trabecular number, thickness and cortical thickness. *compared to control group p<0.05, **compared to control group p<0.01

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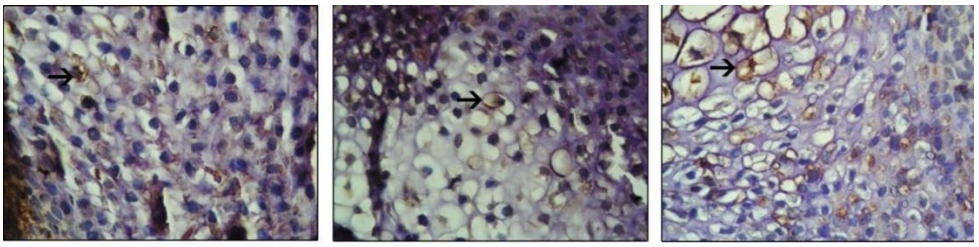

Figure 5a Figure 5b Figure 5c

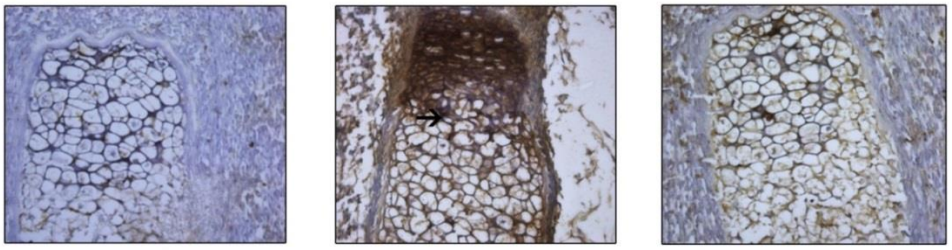

Figure 5d Figure 5e Figure 5f

Figure-5. Immunohistochemistry expressions of β-catenin and S100 in epiphyseal growth plate. β-catenin immunoreactivity X100 **a.** Control group. **b.** Experimental group. **c.** Sham group. S100 immunoreactivity X40 **d.** Control group. **e.** Experimental group. **f.** Sham group. black arrow: positive immunostaining.

Trabecular numbers and thickness: Trabecular numbers were lower in experimental group than control and sham groups (p<0.01 for each). Trabecular thickness was reduced in the experimental group (16.67±3.79) than in the control (37±5.05) and sham groups (24.5±2.12) (p<0.05 for each) (Figure-3a-c, Table-2).

Cortical thickness: Cortical thickness of experimental group (17.46±3.52) was lower than the control (36.07±8.43) and sham groups (21.64±7.51) but no statistical significance was found in these changes (p>0.05 for each) (Table-2).

Immunohistochemical expression of β-catenin and S-100 in growth plate

Examination of β-catenin immunoreactivity in growth plates during intrauterine growth retardation were demonstrated (Figure 4a, b, c). Immunoreactivity of β-catenin was lower in experimental group (+1, weak) compared to the control and sham groups (+2, moderate). According to the results of the S100 immunoreactivity, higher levels of immunoreactive S100 were observed in experimental group (+2, moderate) compared to those in control and sham groups (+1, weak) (Figure-4d, e, f).

Discussion

In this study, we used bilateral uterine artery ligation model of uteroplacental insufficiency to demonstrate that Wnt/β-catenin and S100 expression immunohistochemistry and histomorphometrically on growth plate and bone tissue of fetal rats. Intrauterine growth retardation is very common all over the world, because 13.7 million infants are born with low birth weight

every year. Statistics show that 11% of IUGR cases worldwide are located in developing countries, so intrauterine growth retardation is estimated to be 6 times higher in developing countries (28). For this reason, IUGR studies are extremely important. Poor nutrition and uteroplacental insufficiency, related to IUGR, cause maternal malnutrition during pregnancy period. Previous study showed that, the surgical bilateral uterine artery ligation technique represents a more suitable model to study placental insufficiency (29). Placental nutrition is the most important factor in intrauterine life because fetal development and growth depend on oxygen and glucose. When intrauterine restrictions occur, fetal growth breaks down which may lead to increased perinatal mortality and morbidity. Additionally, intrauterine growth retardation is a risk factor of metabolic syndrome, growth retardation, ischemic heart disease and skeletal abnormalities (2, 4, 30, 31).

The growth plate is shaped as a result of proliferation, differentiation of stem cells and chondrocytes. Especially, the growth and differentiation of stem cells in the reservation zone results in unidirectional bone growth. Hypertrophic cells occur as a result of cellular changes in the proliferation and hypertrophic zone. After the formation of hypertrophy, porliferation shut down in this zone (32, 33). Moreover, the number of hypertrophic chondrocytes is reduced, it leads to reduction in growth plate height claim that chondrocyte proliferation, hypertrophy and longitudinal growth of long bone are fundamentally affected by one another (34, 35). If any hazardous changes occur during this process; thickness of growth plate will be reduced leading to reduced bone formation (35). Thickness of growth plate correlates directly with longitudinal bone growth (11, 36). Histomorphometrical results of this study suggest that the length of growth plate and thickness of its zones was decreased in the experimental group compared to control and sham groups. The reduction of the thickness of the growth plate zones is associated with cartilage cells and different cell types located in these zones. Our results are consistent with the previous studies.

In our study, we show that a distinct decrease in the thickness and number of trabecula occurs after performing of uterine artery ligation in the experimental group. Trabecular architecture affects bone mass and bone quality (5). Tomaszewska et al. claimed that when this architectural structure was destroyed in peri- and postnatal life, bone will be very fragile in the adult life. Trabecular number indicates the quantity of mineralized bone (5). The deficiency of bone trabeculae is related to cartilage structure (37). Vascular organizations and the nutrition in the postnatal life, provided from this vascular organization, are the most important factors for chondrogenesis and osteogenesis. Moreover, this vessel development and nutrition supply are necessary for homeostasis of bone (35).

WNT/β-catenin stimulates the growth plate chondrocytes in chondrogenesis (33, 34). Moreover, β-catenin has major effects in the differentiation of MSCs to chondroblast and osteoblast and proliferation of these cells in osteogenesis (14, 15, 40, 41, 42). Tamamura claims that β-catenin levels have an important role in skeletogenesis. Wnt/β-catenin signaling pathway is effective on chondrocyte behavior and maturation in developmental process in normal skeletal development (9, 36). Previous studies claim that expression of multiple Wnt/β-catenin signaling components have been detected in the growth plate cartilage, especially in prehypertrophic and hypertrophic chondrocytes (16). Moreover, when the Wnt expression is blocked in osteoblast, newly formed bone mass and the thickness of hypertrophic zone decreases in the growth plate (16, 39). Golovchenko et al., showed that rate of trabecular bone architecture in developing stages related to levels of β-catenin expression in chondrocytes in the hypertrophic zone (37). In our study, in experimental group, β-catenin immunoreactivities were lower than those in the other groups.

Previous studies showed that different zones of the growth plate express S100 protein in different ratios. Chondrocytes present in hypertrophic and degeneration zones express more S100 protein. At the same time, it has been found that cartilage cells located in the regions where cartilage damage occurs are expressed more S100 because it has important effects on early cartilage matrix synthesis and repair (20, 43). In our study, increasing expression of S100 in the experimental group indicates that cartilage cells produce matrix proteins for repair after uterine artery ligation.

Chondrogenesis and osteogenesis, is a complex and multi-component process, is affected by many factors as well as placental insufficiency. Disclosure of the molecular mechanism of damage in growth zones and bone architecture in growth retardation due to placental insufficiency will enable the development of new treatment methods.

Conclusion

We conclude that uterine artery ligation leads to shortness of growth plate and degenerated bone architecture because of reduced Wnt/β-catenin signaling.

Conflict of interests

There is no conflict of interests regarding the publication of this paper for the authors. All authors have read and approved the final version of the article.

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