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Effects Of Folic Acid Versus Nicotine On Bone Development

Kemik Gelişiminde Nikotine Karşı Folik Asitin Etkileri

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ÖZ

Amaç: Gebelikte maruz kalınan nikotin sadece anneye değil, fetal dokulara da doğrudan veya dolaylı olarak zarar verir. Bu çalışmadaki amaç gebelik döneminde kullanılan nikotine karşı verilen folik asitin fetusların kemik gelişimine olası etkilerinin araştırılmasıdır.
Araçlar ve Yöntem: 18 yetişkin dişi sıçan kontrol, düşük doz nikotin (DDN), yüksek doz nikotin (YDN), düşük doz nikotin + folik asit (DDN + FA), yüksek doz nikotin + folik asit (YDN + FA) ve folik asit (FA) gruplarına eşit olarak ayrıldı. 20 gün boyunca 1 ml/kg serum fizyolojik (SF) solüsyonu kontrol grubuna, 3 mg/kg nikotin DDN'ye, 6 mg/kg nikotin YDN'ye, 3 mg/kg nikotin ve 400 µg/kg FA DDN+FA'ya, 6 mg/kg nikotin ve 400 µg/kg FA YDN+FA'ya, 400 µg/kg FA FA grubuna uygulandı. Gebeliğin 20. gününde sezaryen ile alınan fetüslerın kemikleri ikili iskelet boyama tekniği ile boyandı. Boyanan ön ve arka ekstremite kemikleri stereomikroskop altında fotoğraflandı. Kemik uzunluğu, kemikleşme derecesi ve kemikleşme yüzdesi ölçüldü. İstatistiki veriler R programlama dili (v. 3.2.3) kullanılarak değerlendirildi.
Bulgular: Kemik gelişimi DDN ve YDN gruplarında anlamlı derecede düşük; DDN+FA ve YDN+FA gruplarında kontrol grubuna yakındı ($p<0.05$).
Sonuç: Gebelikte kullanılan nikotin fetüslerin kemik gelişimini azaltırken, FA bu etkiyi azaltarak kemikleşmeyi arttırabilir.

Anahtar Kelimeler: boyama; iskelet; kemikleşme; sıçan

ABSTRACT

Purpose: Nicotine exposure during pregnancy directly or indirectly harms not only the mother but also the fetal tissues. The aim of this study is to investigate the possible effects of folic acid given against nicotine used during pregnancy on bone development of fetuses.
Materials and Methods: 18 adult female rats were divided into control, low-dose nicotine (LDN), high-dose nicotine (HDN), low-dose nicotine + folic acid (LDN + FA), high-dose nicotine + folic acid (HDN + FA), and folic acid (FA) group equally. During 20 days, 1 ml/kg serum physiologic (SP) solution to the control group, 3 mg/kg nicotine to LDN, 6 mg/kg nicotine to HDN, 3 mg/kg nicotine and 400 µg/kg FA to LDN+FA, 6 mg/kg nicotine and 400 µg/kg FA to HDN+FA, 400 µg/kg FA to the FA group was administered. Bones of fetuses taken by cesarean section on the 20th day of pregnancy were stained with the bilateral skeleton staining technique. The stained anterior and posterior extremity bones were photographed under a stereomicroscope. Bone length, extent of ossification and percentage of ossification were measured. Statistical data were evaluated using the R programming language (v. 3.2.3).
Results: The bone development of LDN and HDN groups was significantly lower and LDN+FA and HDN+FA groups was close to the control group ($p<0.05$).
Conclusion: While nicotine used during pregnancy decreases the bone development of fetuses, FA may decrease this effect and increase ossification.

Keywords: ossification; rats; skeleton; staining

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INTRODUCTION

Pregnant women are exposed to certain chemicals due to living conditions.[1] These chemicals can lead to congenital malformations by getting into the fetal circulation and having various teratogenic effects on the embryo as well as stopping skeletal development which is an important part of somatic growth and development.[2]

One of the most important non-contagious risk factors that threaten human health is cigarette smoke which comprises more than 4800 chemicals, and nicotine is a crucial substance in it.[3] Nicotine is a toxic alkaloid found in the tobacco plant of the Solanaceae family that leads to addiction.[4]

Prenatal exposure to nicotine is an important risk factor that increases the incidence of illness and death for the newborn.[5] Nicotine prevents bone formation by decreasing osteoblast activity, increases osteoclast activity and causes osteonecrosis and periodontal bone loss.[6,7] It reduces the regeneration of fibroblasts and macrophages and delays scar tissue formation.[8] It also decreases vitamin D storage in the liver by altering the metabolism of vitamin D, which is important for bone formation and development.[9]

Folic acid (FA), also known as B9 and folate, is a water-soluble B vitamin. Humans and other mammals cannot synthesize FA in their tissues.[10] According to the World Health Organization (WHO), the daily FA intake for non-pregnant women and adults is 170 µg/kg/day while it is 370-470 µg/kg/day during pregnancy and 270 µg/kg/day during lactation. Women planning a pregnancy should be taking 400 µg/kg/day FA supplementation every day until the 12th week of pregnancy.[11] Inadequate intake of vitamin FA during pregnancy leads to megaloblastic anemia characterized by larger red blood cells stored in the bone marrow than normal.[12]

The effects of folic acid on the possible effects of nicotine on bone development have not yet been in studies. The aim of this study is to investigate role of folic acid against the nicotine on fetal bone development by means of the double staining technique

MATERIALS and METHODS

Selection and Breeding of Experimental Animals

In this study, which was initiated with the approval of Erciyes University Animal Experiments Local Ethics Committee (Date: 09.12.2015 and numbered 15/151), 18 Wistar-Albino female rats with an average weight of 150 g, obtained from Erciyes University Experimental Research and Application Center (DEKAM) were used.

During the study, the rats were kept in DEKAM at a constant temperature of 22 °C in the rooms where 12 hours of light and 12 hours of darkness were provided. The rats were fed with pellet type feed containing 21% crude protein and tap water which they could drink without restriction.

Two rats were randomly placed in the same cage with one male rat at 17.00 on the day of breeding. The following morning at 07.00, female rats underwent a vaginal smear test. Females observed with sperm under microscope were accepted as 0.5 days pregnant and 6 groups (3 rats in each group) were randomly placed in different cages.

Preparation and Application of Nicotine and Folic Acid

SP (1 ml/kg per day) was intraperitoneally (ip.) applied to the rats in the control group.

Nicotine (3 mg/kg per day) was subcutaneously (sc.) applied to LDN.

Nicotine (3 mg/kg per day) was applied subcutaneously and half-hour later FA (400 µg/kg per day) intraperitoneally to LDN+FA.

Nicotine (3 mg/kg per day)was applied subcutaneously twice per day to HDN.

Nicotine (3 mg/kg per day) was applied subcutaneously twice per day and half-hour later FA (400 µg/kg per day) intraperitoneally to HDN+FA.

FA (400 µg/kg per day) was applied intraperitoneally to FA.

In the preparation of nicotine; 19.9 ml of SP was added to 0.1 ml of Nicotine For Synthesis (Merck, M820877.0025), which contains 1000 mg of nicotine in 1 ml. From the resulting 20 ml mixture, 0.09 ml was taken and applied as low dose in the evenings, and 0.09 ml as high dose in the mornings and evenings.

FA was applied according to the recommendation of WHO, which is 400 µg/kg/day. Considering that the rats were on average 150 g, 0.1 ml SF was mixed into 60 µg FA (Sigma, F7876-10). The mixture was prepared daily.

Euthanasia of Rats and Removal of Fetuses

On the 20th day of pregnancy, euthanasia was performed using ketamine (75 mg/kg) + xylazine (10 mg/kg) as anesthetic agent. The anterior abdominal walls of the rats were removed and the fetuses were removed with their placentas. The fetuses were humanely sacrificed using high-dose anesthesia. Fifteen fetuses were taken randomly from each group for measurements.

Staining

Double staining is a technique used in teratogenic and developmental studies that is based on the coloring of the bone and cartilage, which form the skeleton with either different dyes and different colors or different shades of the same color.[13] The first publishing by Simons and Van Horn assured the staining of the bone tissue with Alizarin Red-S and the cartilage tissue with Alcian Blue, after which the double staining technique of the skeleton began to be an often preferred method in 1971.[14] In 1976, Inouye mixed Alizarin Red-S and Alcian Blue into a single solution and stained bone and cartilage tissues at the same time. This method has since been a gold standard in the double staining technique for its quickness and effectiveness.[15]

The fetuses were skinned and the internal organs and eyes were removed before staining. All fetuses were kept in 70% ethanol for 7 days for dehydration. Subsequently, they were kept in pure acetone for 3 days and then cleared of adipose tissue taken into the dye solution in glass containers.

The glass containers, which were cut off from air, were kept in a drying-oven at 37 °C for seven days and the bone and cartilage were stained. At the end of day 7, the fetuses were kept under running tap water for two hours. Then, the transparency phase was initiated. Fetuses which had been stained waited during the 4-stage transparency phase;

1 day in 1% KOH in stage 1.

5 days in a mixture of 1% KOH (80 ml) and 20% glycerin (20 ml) in stage 2.

5 days in a mixture of 1% KOH (50 ml) and 50% glycerin (50 m) in stage 3.

5 days in a solution of 1% KOH (20 ml) and 80% glycerin (80 ml) in stage 4.

The fetuses that underwent the transparency stage were placed into 100% glycerin (Figure 1).

Figure 1. The fetuses in 100% glycerin.

Measurements

The anterior and posterior extremities of the fetuses were separated by forceps and photographs were taken with a Nikon™ E5700 camera. The photographs were transferred to a computer and the bone length, extent of ossification and percentage of ossification were measured using Image J software (Figure 2).

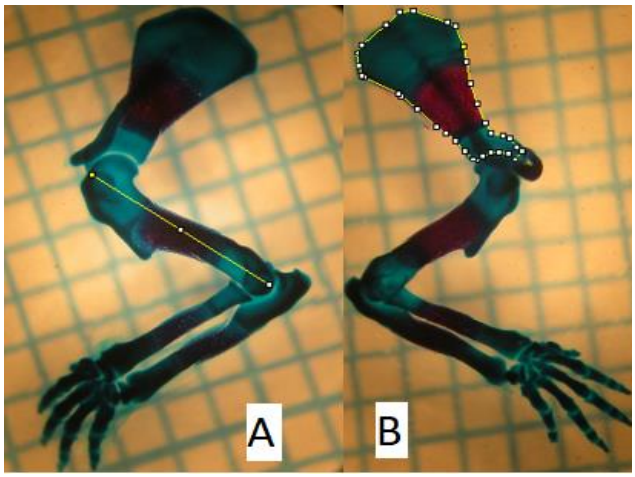

Figure 2. Measurements Using Image J Software.
A. Length Measurement. B. Area Measurement.

Statistical Analysis

Normal distribution of the data was analyzed using Shapiro-Wilk test, histogram and Q-Q plots, whereas homogeneity of variance was examined using Levene's test. One-way analysis of variance (ANOVA) was used for normally distributes intergroup comparisons, and Tamhane's T2 was used in multiple comparisons. Data were analyzed using author-written codes in R programming language version 3.2.3 (release date: December, 2015; URL: https://cran-archive.r-project.org/bin/windows/base/old/3.2.3/). $p<0.05$ was considered statistically significant.

RESULTS

The first effect in nicotine-administered rats was the stage of shock. After this phase, severe convulsions involving the whole body were observed. Following the spasms that lasted approximately 2 minutes, a sedation phase of 2-3 minutes was observed.

a. Measurement Results of Anterior Extremities

The length of scapula, humerus, radius, ulna bones; the extent of the ossification area and the percentage of ossification in the LDN and HDN groups were statistically smaller ($p<0.05$) in comparison with the control group (HDN<LDN<Control).

The length of scapula, humerus, radius, ulna bones; the extent of the ossification area and the percentage of ossification in LDN+FA were statistically bigger ($p<0.05$) in comparison with LDN.

The length of scapula, humerus, radius, ulna bones; the extent of the ossification area and the percentage of ossification in HDN+FA were statistically bigger ($p<0.05$) in comparison with HDN.

The length of scapula, humerus, radius, ulna bones; the extent of the ossification area and the percentage of ossification in FA were smaller in comparison with the control group; however, the difference between them was not statistically significant (Figure 3).

The data regarding anterior extremities bones are given in Table 1 and Table 2.

Table 1. Data regarding Scapula and Humerus Bones.

Variables	Scapula Bones			Humerus Bones		
Groups	BL (mm)	EO (mm^2)	PO (mm^2)	BL (mm)	EO (mm^2)	PO (mm^2)
Control	3.31	1.65	45.7	4.35	1.81 ^{d}	37.97
LDN	3.16 ^{a,b,c,d}	1.38 ^{a,b,c,d}	37.64 ^{a,b,c,d}	4.11 ^{a,d}	1.48 ^{a,b,d}	34.51 ^{a,d}
LDN+FA	3.33	1.66	46.13	4.18	1.74 ^{d}	38.35
HDN	3.12 ^{a,b,c,d}	0.84 ^{a,b,c,d,e}	18.86 ^{a,b,c,d,e}	4.08 ^{a,d}	0.86 ^{a,b,c,d,e}	18.34 ^{a,b,c,d,e}
HDN+FA	3.26	1.55 ^{d}	40.64 ^{a,b,d}	4.14 ^{a,d}	1.57 ^{a,b,d}	37.1 ^{d}
FA	3.3	1.71	48.38	4.34 ^{c,e}	1.9	39.68

Average values were used
BL: Bone lenght, EO: Extent of ossification, PO: Percent of ossification,
(Significant compared to) a: the Control b: the LDN+FA c: the HDN+FAd: the FA e: the LDN.

Table 2. Data regarding Radius and Ulna Bones.

Variables	Radius Bones			Ulna Bones		
Groups	BL (mm)	EO (mm^2)	PO (mm^2)	BL (mm)	EO (mm^2)	PO (mm^2)
Control	3.21	1.35	39.99 ^{b,d}	4.23	1.57	34.58 ^{b,c,d}
LDN	3.08 ^{a,d}	1.15 ^{a,b,c,d}	32.57 ^{a,b,c,d}	4.09 ^{b,d}	1.27 ^{a,b,c,d}	26.1 ^{a,b,c,d}
LDN+FA	3.2	1.31	48	4.24	1.43 ^{d}	41.25
HDN	3.05 ^{a,d}	0.6 ^{a,b,c,d,e}	17.57 ^{a,b,c,d,e}	4.05 ^{a,b,c,d}	0.71 ^{a,b,c,d,e}	14.45 ^{a,b,c,d,e}
HDN+FA	3.14	1.27 ^{d}	39.52 ^{b,d}	4.14 ^{b,d}	1.39 ^{a,d}	38.19 ^{d}
FA	3.26	1.4	47.49	4.29	1.58	42.72

Average values were used
BL: Bone lenght, EO: Extent of ossification, PO: Percent of ossification,
(Significant compared to) a: the Control b: the LDN+FA c: the HDN+FAd: the FA e: the LDN.

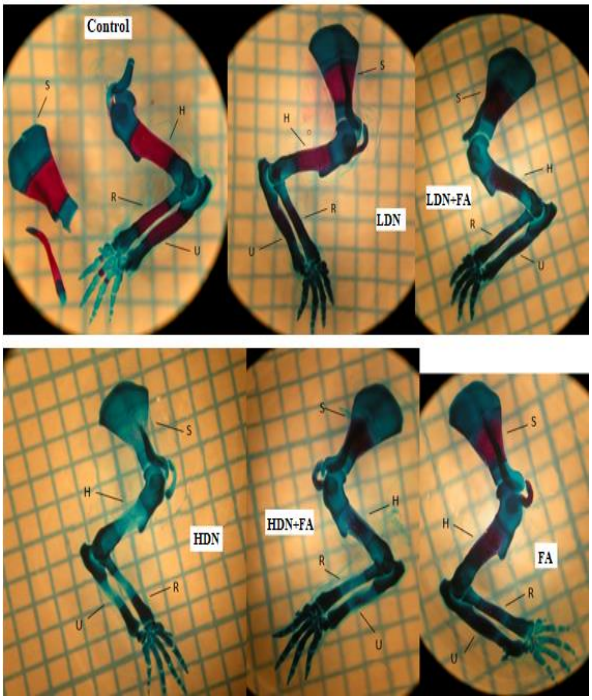

Figure 3. Anterior Extremities Bones.
S: Scapula H: Humerus R: Radius U: Ulna LDN: Low dose nicotine
HDN: High dose nicotine FA: Folic acid.

b. Measurement Results of Posterior Extremities

The length of femur, tibia, fibula bones; the extent of the ossification area and the percentage of ossification in the LDN and HDN groups were statistically smaller ($p<0.05$) in comparison with the control group (HDN<LDN<Control). Ossification in femur, tibia and fibula was not at all observed in the HDN group.

The length of femur, tibia, fibula bones; the extent of the ossification area and the percentage of ossification in LDN+FA were statistically bigger ($p<0.05$) in comparison with LDN.

The length of femur, tibia, fibula bones; the extent of the ossification area and the percentage of ossification in HDN+FA were statistically bigger ($p<0.05$) in comparison with HDN.

The length of femur, tibia, fibula bones, ulna bones; the extent of the ossification area and the percentage of ossification in FA were smaller in comparison with the control group; however, the difference between them was not statistically significant (Figure 4).

The data regarding posterior extremities bones are given in Table 3.

Table 3. Data regarding posterior extremities.

Variables	Femur Bones			Tibia Bones			Fibula Bones		
Groups	BL (mm)	EO (mm^2)	PO (mm^2)	BL (mm)	EO (mm2)	PO (mm^2)	BL (mm)	EO (mm^2)	PO (mm^2)
Control	3.77	1.34	22.54	3.72	1.35	35.29	3.68	1.26	55.77
LDN	3.55 a,d	0.86 a,b,c,d	22.86	3.49 a,d	1.03 a,b,d	22.63 a,d	3.48 a,d	1.05 a,b,c,d	30.73 a,b,c,d
LDN+FA	3.69	1.28 a,d	22.23	3.61	1.22 a,b,d	18.49 a,d,e	3.59 d	1.25	41.81 a,d
HDN	3.45 a,b,c,d	0 a,b,c,d	0 a,b,c,d	3.4 a,b,d	0 a,b,c,d,e	0 a,b,c,d,e	3.41 a,b,c,d	0 a,b,c,d,e	0 a,b,c,d,e
HDN+FA	3.63 a,d	1.2 a,b,d	21.29 a,d,e	3.47 a,d	1.15 a,d	18.3 a,d,e	3.5 a,d	1.19 a,d	36.74 a,b,d
FA	3.79	1.37	23.49	3.73	1.36	35.32	3.71	1.27	56.08

Average values were used
BL: Bone lenght, EO: Extent of ossification, PO: Percent of ossification,
(Significant compared to) a: the Control b: the LDN+FA c: the HDN+FA d: the FA e: the LDN.

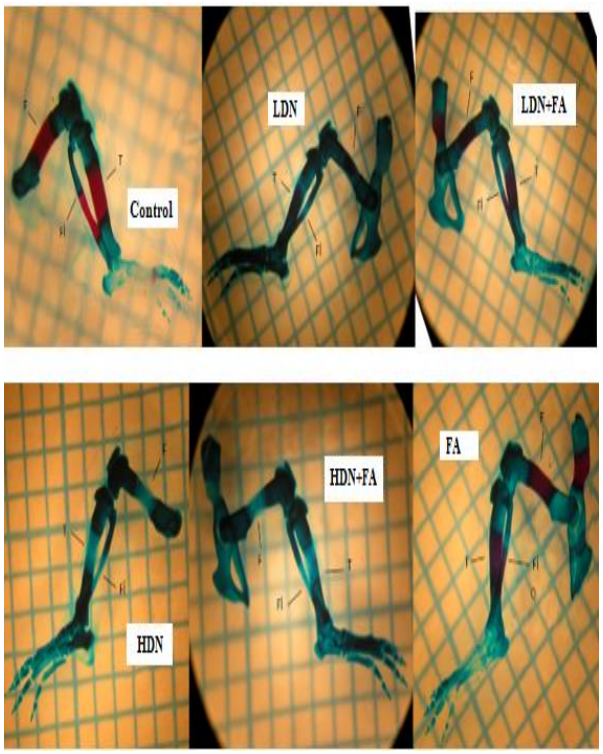

Figure 4. Posterior Extremities Bones.
F: Femur T: Tibia Fi: Fibula LDN: Low dose nicotine HDN: High dose nicotine FA: Folic acid.

DISCUSSION

The main finding of the study is that FA is a powerful antioxidant, and, it has a protective effect against the teratogenic effect of nicotine.

Bone is the basic tissue that is responsible for blood production, adjustment of mineral balance and the movement and protection of the body. It is known that teratogenic substances such as alcohol and cigarette used during pregnancy adversely affect fetal bone development.[16]

To investigate the effects of nicotine on molar teeth in neonatal period was aimed in the fasting study, which involved administering 1.67 mg/kg nicotine intraperitoneally to the mother rats between day 6-21 of pregnancy. On the 4th day after birth, 50 rat pups were weighed and a statistically significant difference between the control group (6.29±0.33 gr) and the nicotine group (4.99±0.32 gr) was found.[17] In our study, a statistically significant difference ($p<0.05$) was found that demonstrated lower weight values between the nicotine-administered LDN group (2.30±0.13 gr) with HDN group (2.13±0.09 gr) and the control group (2.50±0.13 gr).

Patel et al. (2013) conducted a systematic examination by reviewing the orthopedic studies about effects of smoking on bone healing. In their study, they scanned MEDLINE, Web of Science, The Cochrane Library, SCOPUS and EMBASE with the keywords “bone, bone healing, nicotine, tobacco, cigarette” and found that 13 of 17 studies, 9 of which are on tibia and 8 of which are on other orthopedic studies, concluded that smoking adversely affects bone development.[18] In our study analysis of the percentage of tibia bone ossification

indicated that the mean of the control group was 35.29±2.99 mm² and the mean of the LDN group was 22.63±2.46 mm². Ossification in tibia bones was not at all observed in the HDN group.

Mızrak et al. (2014) mixed 0.4 mg/kg/day LDN and 6 mg/kg/day nicotine HDN into the drinking water of rats for 12 months, and gave normal drinking water to the control group. At the end of 12 months, they found that there was no significant difference in the comparison of bone mineral density measurements of the femur and lumbar vertebrae with the control group.[19]

Susar et al. (2017) focused on the protective role of vitamin E against the teratogenic effect of nicotine in their studies. High-Dose Nicotine group they formed were given 6 mg/kg nicotine subcutaneously and the ossification percentage of the bones was examined. It was consequently found that the values were lower than those of the control group.[20] In our study, percentage of ossification in scapulae (18.86±8.12 mm²) in the HDN group was statistically significantly lower in comparison with the control group (45.70±4.57 mm²) as well as lower percentage of ossification in humeri (18.34±8.84 mm²) than that of the control group (37.97±3.41 mm²), lower percentage of ossification in ulnae (14.45±9.78 mm²) than that of the control group (34.58±3.41 mm²) and lower percentage of ossification in radii (17.57±12.89 mm²) than that of the control group (39.99±7.23 mm²).

Vitamin FA plays an important role in the synthesis of DNA and is necessary for maternal health during pregnancy and the normal development of the fetus.[21] Governments and health organizations around the world since the mid-1990s to the present day has emphasized that women should take FA supplements before and during pregnancy to reduce the likelihood of having a baby with brain and spinal tube defects.[22]

Mohammadi et al. (2012) investigated the effect of cyclosporine on bone volume and bone density and whether FA has a protective effect against bone loss. In their experimental studies, 40 Sprague-Dawley rats were randomly divided into 5 groups and for 6 weeks, gave olive oil for the rats in Group A, cyclosporine for the rats in Group B, FA for the rats in Group C, and cyclosporine+FA for the rats in Group D. Group F was designated as the control group. At the end of the study they concluded that the values of Group B (46.3±13.6 mm3) were significantly lower than Group C (80.4±15.70 mm3) and Group D (73.9±21.3 mm3) in the comparison of total volume values of the mandible. Mohammadi et al. concluded that an increase in cyclosporine decreases bone mineral density and causes bone loss; FA may have a protective effect against bone loss.[23]

This study, therefore, examines the protective role of folic acid against the teratogenic effect of nicotine on embryonic bone development. As a result of this study, it was found that nicotine use impaired bone development depending on the dose. Scapula, ulna, radius and fibula bones were most affected by LDN; and humerus, femur and tibia bones were less affected by LDN. The developmental impairment of the anterior bones was higher in HDN in comparison with LDN, and the development of posterior bones was completely prevented in HDN; whereas FA, which was administered in addition to LDN and HDN, played a protective role by increasing bone development.

Conflict of Interest

The authors declare that there is not any conflict of interest regarding the publication of this manuscript.

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Ethics Committee Permission

Approval was received for this study from Erciyes University Animal Experiments Local Ethics Committee (Date: 09.12.2015 and numbered 15/151).

Authors' Contributions

Concept/Design: KD, MN, AP, TE, HG, ÖA. Data Collection and/or Processing: KD, MN, AP, TE, HG, ÖA. Data analysis and interpretation: KD, MN, AP, TE, HG, ÖA. Literature Search: KD, AP. Drafting manuscript: KD, MN. Critical revision of manuscript: KD, MN, TE. Supervisor: MN, TE, HG, ÖA.

REFERENCES

1. Yılmaz H, Ertekin T, Atay E, et al. Antioxidant role of melatonin against nicotine's teratogenic effects on embryonic bone development. Iran J Basic Med Sci. 2018;21(8):787-793.
2. Nisari M, Ulger H, Unur E, Karaca O, Ertekin T. Effect of interleukin 12 (IL-12) on embryonic development and yolk sac vascularisation. Bratisl Lek Listy. 2014;115(9):532-537.
3. Lugg ST, Scott A, Parekh D, Naidu B, Thickett DR. Cigarette smoke exposure and alveolar macrophages: mechanisms for lung disease. Thorax. 2022;77(1):94-101.
4. Callahan PM, Terry AV Jr, Peitsch MC, Hoeng J, Koshibu K. Differential effects of alkaloids on memory in rodents. Sci Rep. 2021;11(1):9843.
5. Yüce B, Tengiz Fİ. Effects of tobacco use during pregnancy on infant and child health. D J Med Sci. 2020;6(2):70-73.
6. Marinucci L, Bodo M, Balloni S, Locci P, Baroni T. Subtoxic nicotine concentrations affect extracellular matrix and growth factor signaling gene expressions in human osteoblasts. J Cell Physiol. 2014;229(12):2038-2048.
7. Kirschneck C, Proff P, Maurer M, Reicheneder C, Römer P. Orthodontic forces add to nicotine-induced loss of periodontal bone: An in vivo and in vitro study. J Orofac Orthop. 2015;76(3):195-212.
8. Payas A, Ekinci Y, Gürbüz K, et al. Vitamin B12 reduces the negative effects of nicotine on fetal bone development in the rats. Jt Dis Relat Surg. 2022;33(1):216-224.
9. Manavi KR, Alston-Mills BP, Thompson MP. History of tobacco, vitamin D and women. Int J Vitam Nutr Res. 2020;90(5-6):389-394.
10. Shulpekova Y, Nechaev V, Kardasheva S, et al. The Concept of Folic Acid in Health and Disease. Molecules. 2021;26(12):3731.
11. Vajda FJE, O'Brien TJ, Graham JE, et al. Folic Acid dose, valproate and fetal malformations. Epilepsy&Behavior. 2021;114:107569.
12. Green R. Vitamin B12 deficiency from the perspective of a practicing hematologist. Blood. 2017;129(19):2603-2611.
13. Booth M, Powell N, Corfield C, French JM. An automated technique for double staining of bone and cartilage in fetal mouse skeletal specimens using alizarin red S and Alcian blue. Biotech Histochem. 2022;97(3):222-227.
14. Dingerkus G, Uhler LD. Enzyme Clearing Of Alcian Blue Stained Whole Small Vertebrates For Demonstration Of Cartilage. Stain Technol. 1977;52(4):229-232.
15. Liao YJ, Tang PC, Chen LR, Yang JR. A protocol for differential staining of cartilages and ossified bones in fetal and adult mouse skeletons using alcian blue and alizarin red S. J Histotechnol. 2020;43(4):204-209.
16. Çetin E, Malas MA. Fetal Büyümeye Etki Eden Çevresel Faktörler. S.D.Ü. Tıp Fak. Derg. 2005;12(2):65-72.
17. Oruç Ş. Ratlarda Hamilelik Döneminde Verilen Nikotinin, Neonatal Dönemde Molar Dişler Üzerindeki Etkileri, Master Thesis, Dicle Üniversitesi; 1996. https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=91qsG18yUN59V_N5VMHDKw&no=91qsG18yUN59V_N5VMHDKw. Accessed 01 April, 2023.
18. Patel RA, Wilson RF, Patel PA, Palmer RM. The Effect of Smoking On Bone Healing. Bone Joint Res. 2013;2(6):102-111.
19. Mızrak S, Turan V, Inan S, et al. Effect of Nicotine on RANKL and OPG and Bone Mineral Density. J Invest Surg. 2014;27(6)327-331.
20. Susar H, Aycan K. Nikotinin Embriyonal Kemik Gelişimi Üzerindeki Teratojenik Etkisine Karşı E Vitamininin Koruyucu Rolü, Phd Thesis, Erciyes University, 2017. https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=qiHiezOEio-GiML-DvHTy9w&no=B387_zfwUqbAIMeYXZJF6A. Accessed 01 April, 2023.
21. Canever L, Alves CS, Mastella G, et al. The Evaluation of Folic Acid-Deficient or Folic Acid-Supplemented Diet in the Gestational Phase of Female Rats and in Their Adult Offspring Subjected to an Animal Model of Schizophrenia. Mol Neurobiol. 2018;55(3):2301-2319.
22. Al-Gailani S. Making birth defects 'preventable': preconceptional vitamin supplements and the politics of risk reduction. Stud Hist Philos Biol Biomed Sci. 2014;47:278-289.
23. Mohammadi A, Omrani L, Omrani LR, et al. Protective Effect of Folic Acid on Cyclosporine-Induced Bone Loss in Rats. Transpl Int. 2012;25(1):127-133.