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Araştırma Makalesi / Research Article

The protective role of vitamin E against teratogenic effects of nicotine on embryonic bone development

Nikotinin embriyonik kemik gelişimi üzerindeki teratojenik etkilerine karşı E vitamininin koruyucu rolü

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Abstract

Aim: According to World Health Organization data, around 1.5 billion people in the world use tobacco products. Nicotine, the most common use of tobacco, is the main psychoactive substance that causes addiction. Exposure to nicotine during pregnancy increases the risk of low placenta weight, stillbirth, congenital heart disease, musculoskeletal defect. Antioxidants are used to protect against teratogenic substances such as nicotine. The purpose of the study was to determine the skeletal system malformations caused by low (3 mg/kg) and high (6 mg/kg) doses of nicotine during embryonic bone evolution by using the double skeletal staining method and the protector role of vitamin E in preventing these malformations.

Material and Method: The rats were divided into 6 groups: the control, low-dose nicotine, high-dose nicotine, low-dose nicotine+vitamin E, high-dose nicotine+vitamin E and vitamin E. The development of the skeletal system of the fetuses was examined by the skeleton staining method. The anterior and posterior extremity images of the fetuses were examined under the stereomicroscope and then through photographing total bone length, ossification length and ossification rate were calculated in the ImageJ program.

Results: There was an important decline in the total bone length, ossification length and ossification rate ($p<0.05$) in the bone measurements of the front and hind extremities, while it was found that the treatment groups approached the control group and the increases were important ($p<0.05$).

Conclusion: It was concluded that being to nicotine during pregnancy delayed skeletal ossification and that vitamin E, which is an antioxidant, may be protective opposite the teratogenic effect of nicotine on the bone.

Keywords: Bone development, double skeletal staining, nicotine, rat, vitamin E

Öz

Amaç: Dünya Sağlık Örgütü verilerine göre dünyada yaklaşık 1.5 milyar insan tütün ürünleri kullanmaktadır. Tütünün en yaygın kullanımı olan nikotin, bağımlılık yapan başlıca psikoaktif maddedir. Hamilelik sırasında nikotine maruz kalmak, düşük plasenta ağırlığı, ölü doğum, doğuştan kalp hastalığı, kas-iskelet sistemi kusuru riskini artırır. Antioksidanlar, nikotin gibi teratojenik maddelere karşı koruma sağlamak için kullanılır.

Bu çalışmanın amacı, embriyonik kemik gelişimi sırasında düşük (3 mg/kg) ve yüksek (6 mg/kg) doz nikotinin neden olduğu iskelet sistemi malformasyonlarını ikili iskelet boyama yöntemi ile belirleyerek; E vitamininin koruyucu rolünü ortaya koymaktır.

Materyal ve Metot: Çalışmada ratlar; kontrol, düşük doz nikotin, yüksek doz nikotin, düşük doz nikotin+E vitamini, yüksek doz nikotin, yüksek doz nikotin+E vitamini ve E vitamini olmak üzere 6 gruba ayrıldı. Fetüslerin iskelet sistemi ikili boyama yöntemi ile boyandı. Fetüslerin ön ve arka ekstremite görüntüleri stereomikroskop altında incelendikten sonra; ImageJ programında toplam kemik uzunluğu, kemikleşme uzunluğu ve kemikleşme alanları hesaplandı.

Bulgular: Ön ve arka ekstremite kemik ölçümlerinde toplam kemik uzunluğu, kemikleşme uzunluğu ve kemikleşme alanında ($p<0.05$) anlamlı bir düşüş varken, tedavi gruplarının kontrol grubuna yaklaştığı ve artışların anlamlı olduğu saptandı ($p<0.05$).

Sonuç: Gebelikte nikotine maruz kalmanın iskelet kemikleşmesini geciktirdiği ve bir antioksidan olan E vitamininin nikotinin kemik üzerindeki teratojenik etkisine karşı koruyucu olabileceği sonucuna varıldı.

Anahtar Kelimeler : Kemik gelişimi, ikili iskelet boyama, nikotin, sıçan, E vitamini

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INTRODUCTION

Bone is an active tissue that is permanently affected by nutritional, hormonal and metabolic status (1-3). Women throughout pregnancy may be exposed to some chemicals depending on their living experience. These can cause various teratogenic impacts in the embryo (4,5). Smoking is bad habit that has harmful consequences for human health because it includes 7000 toxic chemicals. This habit results in addiction. The main addictive substance in cigarettes is nicotine. The total alkaloid constitutes about 95% of the content and nicotine constitutes about 0.5 to 8.0% of the dry weight of the tobacco. Other alkaloids are cotinine, nornicotine, database and nicotine-N-oxide (6). Only one cigarette generally includes 0.6-2 mg nicotine (7). Nicotine reaches the fetus through the placenta (8). It has been reported that the concentration of nicotine in fetal circulation is 15% higher than the maternal serum and 88% higher than amniotic fluid (9-11).

Passive exposure to smoking or smoking during pregnancy can cause fetal defects (12). Scientific studies have shown that nicotine significantly delays fetal bone development (13). Smoking has a direct effect on bone tissue. Nicotine is an inhibitor of osteogenesis and angiogenesis processes that play a role in bone development (14). It has been shown in the literature that it reduces the storage of vitamin D, which plays a role in bone metabolism, and delays scar tissue (15-17). Several studies have shown that nicotine disrupts the oxidant-antioxidant process. Different studies have reported that antioxidants are beneficial to this deteriorating process (18). Vitamin E refers to tocopherols and tocotrienols which are synthesized by plants and are soluble in fat. It is found in fatty foods with different proportions (19). Studies are showing that vitamin E, a potent antioxidant, has a positive effect on the skeletal system (20). Vitamin E prevents the increase of osteoclast activity by clearing free radicals in the body and keeps the bone remodeling process in balance Vitamin E prevents the increase of osteoclast activity by clearing free radicals in the body and keeps the bone remodeling process in balance. It has been reported in various studies that vitamin E increases bone trabeculae and bone volume, decreases osteoclast activity, and increases osteoblast activity (21).

Skeletal system malformations that may occur due to the use of nicotine during pregnancy can be reduced by taking appropriate doses of vitamin E. The purpose of the study is to define the skeletal system malformations caused by nicotine during embryonic bone development by using the double skeletal staining method and the protective role of vitamin E in preventing these malformations.

MATERIAL AND METHOD

Animal selection

The study was conducted with the decision of the local ethics centre of Erciyes University Experimental and Clinical Research Center dated on 11.11.2015 and decision number 15/143. 18 with grown-up female (5-7 months old) Wistar-Albino rats weighed 180-220g. Female rats and male rats were put in the same lattice. The following morning, samples of vaginal smears received from female rats were examined under the light microscope. Females, whose vaginal smear showed sperm, were accepted as 0.5 days pregnant.

Experimental groups

Ethically, a minimum number of rats and fetuses were used. Six different groups (n=3) were formed from pregnant rats.

Control Group (C): To the rats in this group, 1 ml/kg/day saline was given into the peritoneum (i.p.).

Vitamin E Group (Vit E): To the rats in this group were given 60 mg/kg/day vitamin E administration by i.p.

Low Dose Nicotine Group (LDN): The rats were given 3 mg/kg/day nicotine administration subcutaneous (s.c.)

Low Dose Nicotine+Vitamin E Group (LDN+Vit E): The rats were given 3 mg/kg/day nicotine by s.c. and half an hour after, 60 mg/kg/day vitamin E was administered by i.p.

High Dose Nicotine Group (HDN): Nicotine administration was performed at a rate of 3 mg/kg/day s.c. twice a day, a total of 6 mg/kg/day.

High Dose Nicotine+Vitamin E Group (HDN+Vit E): Nicotine administration was performed at a rate of 3 mg/kg/day s.c. twice a day. Vitamin E was administered 60 mg/kg/day by i.p. half an hour after nicotine administration.

* Every day injection was given to rats in all groups, 1st and 20th day of pregnancy.

* Pregnant rats in all groups were sacrificed on the 20th day of pregnancy.

Preparation of injections

Nicotine with the code N3876 and Vitamin E with the code T3251 as a-tocopherol form were obtained from the Sigma-Aldrich company (Darmstadt, Germany). Saline was used for nicotine solution and olive oil was used for vitamin E solution.

Obtaining fetuses

The fetuses were dissected together with the placentas. The weight of fetuses was weighed with a precision scale. Head and stern lengths were measured with a digital calliper. Obtained data were recorded. Considering the exclusions, in each group, without gender detection, 15 of the fetuses were used for double skeletal staining method. Fetuses used for the double skeletal staining were separately measured on both extremities (15 right front and hind extremity, 15 left front and hind extremity).

Double skeletal staining of fetuses

Fetuses were taken to 70% ethyl alcohol for 4-7 days. After this process, they were kept in acetone for 1-3 days. The internal organs of the fetuses were removed. After acetone, they were taken into a double staining solution prepared with Alizarin Red-S (100 mg) and Alcian Blue (300 mg).

Then they were kept in the oven at 38-40 0C. Afterwards, the tissues of the fetuses were washed with tap water for 2 hours. The transparency phase was started with 1% potassium hydroxide (KOH). Skeletal stained fetuses were preserved in 20%, 50%, 80% and 100% glycerin. For the measurement of fetal extremities, photographs were taken with a Nikon E5700 trademark digital camera under a stereomicroscope. The length and area measurements of the bones were made in ImageJ (http://rsb.info.nih.gov/ij/docs/index.htlm) program. Findings from area measurements were used to determine ossification.

Statistical analysis

Bone and ossification lengths were examined by the ImageJ program. All bone and ossification surface areas were calculated. Data obtained from measurements were analyzed with IBM Statistical Package for the Social Sciences 22 program. The Kolmogorov–Smirnov test was applied to determine the normal distribution of throughputs. A One-Way ANOVA test was done on the data. One-way analysis of variance with the post hoc Tukey honestly significant difference (HSD) test has applied to the differences between the groups. Results of the analysis, it was admitted that there was a meaningful difference between the groups with p values of <0.05.

RESULTS

Effects on growth parameters

Table 1 showed that a statistically meaningful decline in the weights of the fetuses in the experimental groups with low dose (3 mg/kg) and high dose (6 mg/kg) nicotine compared to the control group ($p<0.05$). Table 2 showed that a statistically meaningful decline in head-rump lengths ($p<0.05$). When 60 mg/kg vitamin E was given as a preservative, it was determined that there was a statistically meaningful increase in the growth parameters and the values approached the control group.

Table 1. Weight of fetuses

Fetus No	Weight (g)					
	C	LDN	LDN+Vit E	HDN	HDN+Vit E	Vit E
1	2.15	2.03	2.35	2.06	1.93	2.42
2	2.40	2.15	2.22	1.68	2.05	2.21
3	2.41	2.00	2.58	2.04	1.97	2.23
4	2.20	2.14	2.60	1.56	2.10	2.36
5	2.40	1.89	2.17	2.20	2.21	2.33
6	2.17	2.10	2.25	2.01	2.09	2.35
7	2.24	2.43	2.47	2.05	2.30	2.45
8	2.43	2.05	2.40	1.95	2.38	2.62
9	2.54	2.21	2.07	1.93	2.00	2.20
10	2.27	2.20	2.03	1.98	1.91	2.25
11	2.32	2.26	2.14	1.87	1.86	2.30
12	2.38	2.38	2.23	1.95	2.07	2.43
13	2.65	2.11	2.11	2.01	2.14	2.40
14	2.63	2.18	2.15	2.10	2.20	2.54
15	2.23	2.31	2.27	1.94	2.17	2.24
Mean±SD	2.36±0.15	2.16±0.14a,d	2.26±0.17	1.95±0.15a,b,d,e	2.09±0.14a,d	2.35±0.12

*in the statistical evaluation of all table data; ANOVA test; P<0.05 was considered statistically significant; C: Control, LDN: low-dose nicotine, HDN: high-dose nicotine, Vit E: Vitamin E; (a) It is significant when compared with the control group; (b) It is significant when compared with the LDN+Vit E group; (c) It is significant when compared with the HDN+Vit E group; (d) It is significant when compared with the Vit E group; (e) It is significant when compared with the LDN group

Table 2. Head-rump length of the fetus

Fetus No	Length (mm)					
	C	LDN	LDN+Vit E	HDN	HDN+Vit E	Vit E
1	28.55	25.13	30.15	26.64	25.00	30.32
2	27.74	24.33	28.87	23.56	25.64	29.76
3	29.18	25.17	27.67	23.75	27.17	28.86
4	29.10	27.70	28.51	21.89	27.54	29.27
5	29.52	29.15	27.32	23.47	25.03	31.92
6	28.73	25.17	28.20	23.32	28.67	28.63
7	28.80	26.87	26.76	26.54	27.06	27.69
8	30.32	29.76	27.15	25.44	27.21	28.42
9	31.68	28.04	27.28	22.74	25.45	29.54
10	27.33	25.23	28.11	24.65	26.19	31.01
11	30.78	27.21	27.30	26.58	26.83	29.46
12	31.16	29.18	28.67	22.87	26.05	29.15
13	28.45	27.59	27.92	25.14	28.23	30.35
14	30.12	27.78	30.95	26.02	27.70	27.47
15	29.89	28.54	30.88	25.20	26.43	29.58
Mean±SD	29.42±1.23	27.12±1.73a,b,d	28.38±1.33	24.52±1.55a,c,d,e	26.68±1.12a	29.42±1.17

*in the statistical evaluation of all table data; ANOVA test; P<0.05 was considered statistically significant; C: Control, LDN: low-dose nicotine, HDN: high-dose nicotine, Vit E: Vitamin E; (a) It is significant when compared with the control group; (b) It is significant when compared with the LDN+Vit E group; (c) It is significant when compared with the HDN+Vit E group; (d) It is significant when compared with the Vit E group; (e) It is significant when compared with the LDN group

Findings of front extremity bones

In the study, (humerus, radius, ulna) bones of the anterior extremity were evaluated. The istatistically meaningful decline in ossification length and ossification rate was found in 3 mg/kg nicotine compared to the control group ($p<0.05$). A statistically meaningful decline in ossification data was detected in the 6 mg/kg nicotine treated group compared to the 3 mg/kg given group ($p<0.05$). When nicotine, vitamin E were applied with, ossification raised and approached the control group (Table 3), (Figure 1), ($p<0.05$).

Findings of hind extremity bones

In the study, (femur, tibia, fibula) bones of the posterior extremity were evaluated. A statistically meaningful decline in ossification length and ossification rate was found in 3 mg/kg nicotine compared to the control group ($p<0.05$). A statistically meaningful decline in ossification data was detected in the 6 mg/kg nicotine treated group compared to the 3 mg/kg given group ($p<0.05$). When nicotine, vitamin E were applied with, ossification raised and approached the control group ($p<0.05$), (Table 4), (Figure 2).

Table 3. Ossification rate of the front extremity long bones

		Humerus			Ulna			Radius		
	N	Total bone length	Length of ossified part	Ossification rate (%)	Total bone length	Length of ossified part	Ossification rate (%)	Total bone length	Lengthof ossified part	Ossification rate (%)
Control	30	4.30±0.06	1.86±0.18	44.89±1.69	4.16±0.20	1.92±0.13	45.08±2.75	3.32±0.09	1.58±0.13	43.78±4.53
LDN	30	4.23±0.23	1.55±0.24a,b,d	37.72±6.31a,d	4.07±0.17	1.57±0.32a,b,d	34.45±6.43a,b,d	3.27±0.16	1.30±0.25a,b,d	37.04±4.92a,d
LDN+Vit E	30	4.27±0.21	1.83±0.23	41.55±3.81	4.30±0.29	1.86±0.15	40.17±5.63	3.30±0.13	1.52±0.16	40.40±3.42
HDN	30	3.97±0.23 a,c,d,e	1.17±0.42 a,c,d,e	30.39±13.43 a,c,d,e	3.72±0.20 a,c,d,e	1.12±0.43 a,c,d,e	28.96±14.18 a,c,d	2.98±0.26 a,c,d,e	1.10±0.44 a,c,d,e	32.08±12.11 a,c,d,e
HDN+Vit E	30	4.15±0.16a	1.71±0.20	39.01±2.84a	4.01±0.17	1.75±0.14	38.75±5.89a,d	3.24±0.17	1.41±0.13	39.13±4.05
Vit E	30	4.28±0.17	1.85±0.17	42.70±2.49	4.11±0.13	1.87±0.16	44.97±3.40	3.29±0.07	1.55±0.08	42.43±4.14

*in the statistical evaluation of all table data; ANOVA test; P<0.05 was considered statistically significant; C: Control, LDN: low-dose nicotine, HDN: high-dose nicotine, Vit E: Vitamin E; (a) It is significant when compared with the control group; (b) It is significant when compared with the LDN+Vit E group; (c) It is significant when compared with the HDN+Vit E group; (d) It is significant when compared with the Vit E group; (e) It is significant when compared with the LDN group

Table 4. Ossification rate of the hind extremity long bones

		Femur			Tibia			Fibula		
	N	Total bone length	Length of ossified part	Ossification rate (%)	Total bone length	Length of ossified part	Ossification rate (%)	Total bone length	Length of ossified part	Ossification rate (%)
Control	30	3.71±0.34	1.32±0.17	33.85±4.30	3.75±0.15	1.48±0.25	37.41±5.46	3.55±0.19	1.45±0.13	40.52±5.11
LDN	30	3.52±0.19 a,d	0.96±0.19 a,b,d	25.16±2.51 a,b,d	3.60±0.20d	1.02±0.12 a,b,d	24.46±4.93 a,b,d	3.53±1.77	0.85±0.27	27.86±5.13 a,b,d
LDN+Vit E	30	3.68±0.25	1.31±0.22	32.22±4.83	3.70±0.19	1.38±0.28	33.44±6.91	3.56±0.20	1.20±0.32	36.87±6.57
HDN	30	3.35±0.23 a,c,d	0.70±0.28 a,c,d,e	18.82±8.04 a,c,d,e	3.10±0.40 a,c,d,e	0.75±0.23 a,c,d,e	21.51±11.05 a,c,d,e	3.10±0.17 a,c,d,e	0.60±0.31	22.33±8.90 a,c,d,e
HDN+Vit E	30	3.60±0.18	1.23±0.12	27.31±5.19a,d	3.35±0.20a,d	1.10±0.17a,d	30.38±3.63a,d	3.30±0.18a,d	1.10±0.18	31.70±6.31a,d
Vit E	30	3.72±0.18	1.33±0.09	32.04±4.39	3.80±0.19	1.46±0.18	36.05±2.90	3.62±0.27	1.42±0.20	39.38±4.40

*in the statistical evaluation of all table data; ANOVA test; P<0.05 was considered statistically significant; C: Control, LDN: low-dose nicotine, HDN: high-dose nicotine, Vit E: Vitamin E; (a) It is significant when compared with the control group; (b) It is significant when compared with the LDN+Vit E group; (c) It is significant when compared with the HDN+Vit E group; (d) It is significant when compared with the Vit E group; (e) It is significant when compared with the LDN group

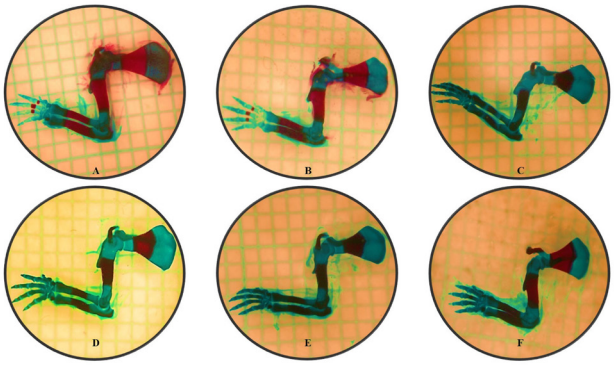

Figure 1. View of the anterior extremity bones. (A) Control group, (B) Low-dose nicotine group, (C) Low-dose nicotine+Vitamin E group, (D) Vitamin E group, (E) High-dose nicotine group, (F) High-dose nicotine+Vitamin E group

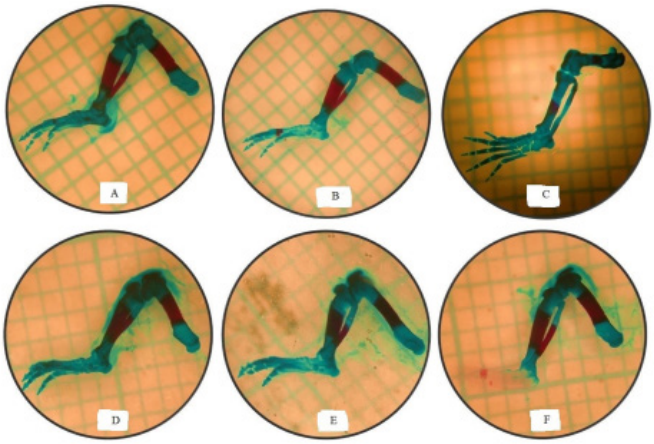

Figure 2. View of the posterior extremity bones. (A) Control group, (B) Low-dose nicotine group, (C) Low-dose nicotine+Vitamin E group, (D) Vitamin E group, (E) High-dose nicotine group, (F) High-dose nicotine+Vitamin E group

DISCUSSION

Nicotine damages both the development and function of the placenta. It was detected that nicotine deteriorates the metabolism and increases oxidative stress in the placenta (22,23). It also increases the destruction of exogenous estrogens by suppressing the activity of osteoblasts. Therefore, it causes low body weight and earlier menopause (24). In this study, in which we aimed to show the protection of vitamin E on the harms of nicotine on fetal development, your most emphatic result is that the ossification of the radius, ulna and femur develops well, close to the control group.

Hakem 2: "Nikotinin fetal gelişim üzerindeki zararlarına karşı E vitamininin koruyuculuğunu göstermeyi amaçladığımız bu çalışmada en önemli sonucumuz radius, ulna ve femur kemikleşmesinin kontrol grubuna yakın bir şekilde iyi geliştiğidir" şeklinde cümle eklendi.

Oruç (25), applied rats to 1.67 mg/kg nicotine by i.p. between 6 and 21 days of pregnancy in 1996. In the study, they weighed 50 offspring in the control group (6.29±0.33 g) and nicotine (4.99±0.32 g) on the 4th day after delivery and they reported that the birth weight of the offspring in the nicotine group was low and this decrease was statistically significant. Also Oruç, reported that in the control group samples, molar teeth showed a normal development while the molar tooth sections of the nicotine group samples showed thinning and demineralization areas in the dentin layer and indicated that nicotine hurt hurmed bone development and thus on tooth development. In our study, there was a statistically meaningful decline in the body weights of fetuses in the control group (2.36±0.15 g) and low-dose nicotine (2.16±0.14 g) and high-dose nicotine (1.95±0.15 g) in the control group (Table 1), ($p<0.05$).

Yazıcı (26) have studied the teratogenic effects of nicotine on growth and development of head and face skeleton with prenatal palate formation in rats; nicotine hydrogen tartrate was started before the pregnancy (14 days before pregnancy and it was left at the zero day of the pregnancy), during pregnancy (nicotine applied until 17th day of pregnancy) nicotine was applied 2.7 mg/kg as i.p. in different groups. When the growth and development of the head and facial skeleton, depending on the nicotine application during pregnancy and before pregnancy, it was reported that the width of the foramen incisive increased, the length was decreased and the length of the corpus mandibulae decreased. As a result of their studies, they reported that nicotine delayed ossification. In our study, we applied low dose (3 mg/kg) and high dose (6 mg/kg) nicotine by s.c. throughout pregnancy. In the rats, we looked at the total length of the front and hind extremity bones, ossification length and ossification surface area. We found a significant decrease in total bone/ossification length and narrowing of ossification surface area ($p<0.05$).

Bastug (27) investigated that the effects of exposure to nicotine on the development of juvenile bone during pregnancy and lactation (until postnatal 21st day). In their study, they administered nicotine 3 mg/kg/day by s.c. In our study, we formed high-dose nicotine and low-dose nicotine groups. Our low-dose nicotine group was given 3 mg/kg/day, as applied by Bastug. As a result of the study of Bastug, it was concluded that the birth weight, epiphyseal and hypertrophic zone thickness of the nicotine group were lower than the other groups. Similarly, in our study, the birth weight was low in the nicotine group. The mean weight of the offspring in our control group was 2.36±0.15 g; the mean weight of the offspring rats in the 3 mg/kg nicotine treated group was 2.16±0.14 g. The difference was statistically significant ($p <0.05$).

Kurtoglu et al. (28) gave 3 mg/kg/day nicotine to rats in the gestational and lactation period. At the end of the study, they looked at the femoral length, bone density with bone content of the fetuses of 21 days. They reported that the birth weight of the offspring of the mothers exposed to nicotine was 5.47±0.39 g, which was lower than the control

group and the values of nicotine groups were meaningful lower in the femoral neck, bone density and content than the control groups. In our study, we found that both the body weight and the femur length of the offspring in the low dose nicotine group (3.52 ±0.19) were significantly shorter than the control group (3.71±0.34), ($p<0.05$).

Farag et al. (29) investigated the effect of chronic nicotine exposure on the bone mineral content of adult and young rats. For 6 months 3-4.5 mg/kg/day nicotine was given to rats as s.c. The weight gain in nicotine-treated rats decreased to the dose compared to the control group. They also reported that the femur weights of rats treated with nicotine were lower than the control group and that the concentration of calcium and phosphorus in femur and lumbar vertebrae decreased significantly. They reported that older rats were more affected by changes. They found that the femoral lengths of the rats treated with nicotine were shorter than the control group. As a result, the negative effect of nicotine on bone was found to be increased with age. In our study, the length of the femur belonging to the low dose group of nicotine was 3.52±0.19 mm and the length of the high dose group of nicotine was 3.35±0.23 mm. Both lengths were shorter than the control group and the difference was statistically significant ($p<0.01$). The total shortening of bone length was found to be increased as the dose exposed to nicotine was increased.

The negative effects of nicotine on bone development have been scientifically introduced and protective antioxidants have been used. One of them is vitamin E. Vitamin E is an antioxidant that can prevent lipid peroxidation (21). It is soluble in oil. There are two forms of it as tocopherol and tocotrienol in nature. Vitamin E prevents the increase of osteoclast activity by clearing free radicals in the body and keeps the bone remodeling process in balance. It also increases bone trabecular density by preventing bone calcium loss (30).

Norazlina et al. (31) investigated the effects of vitamin E supplementation in rats with impaired bone metabolism due to nicotine administration. For 3 months, rats received nicotine as 7 mg/kg by i.p. They gave nicotine to one group of rats with nicotine administered as 60 mg/kg alpha-tocopherol in the last two months and 60 mg/kg in the last two months. They looked at serum interleukin-1 (IL-1) and interleukin-6 (IL-6), osteocalcin and bone calcium levels. They also made evaluations in the left femur and the fourth lumbar vertebra. As a result, they indicated that increased levels of IL-1 and IL-6 due to nicotine was turned back after the addition of vitamin E and there was no change in osteocalcin level. Calcium levels were unchanged in femoral bone, whereas bone calcium levels in lumbar vertebrae were lower than in the control group.

Hermizi et al. (32) studied the beneficial effects of vitamin E forms on bone histo-morphometrically parameters after discontinuation of nicotine. After giving nicotine to one of the groups in which they gave 7 mg/kg nicotine for 2 months, they gave alpha-tocopherol and the other had given gamma-tocotrienol (60 mg/kg). At the end of a total of 4 months, they looked at trabecular bone volume, bone mineral ratios and osteoclast amount. They stated that the negative effects of nicotine were reversed in the groups with vitamin E added. As a result, they concluded that vitamin E could be used therapeutically as bone damage in chronic smokers. In our study, we used 60 mg/kg alpha-tocopherol to be protective against nicotine and we found a statistically significant increase in ossification parameters (bone height, ossification length, ossification surface area) ($p<0.05$).

In the study of Soysal et al. (2), the effects of phenytoin, folic acid and vitamin E on bone development were investigated by dual skeletal staining in rat fetuses. The average head and stern length of the fetuses in the control group were found to be 3.21±0.27 mm and their weight was 3.51±0.35 g. They found that the phenytoin groups had shortened the length of the fetus and found a decrease in their weight. They reported a statistically significant increase in these parameters in vitamin E supplemented experimental groups. These findings showed that the vitamin E which we used against the teratogenic effect of nicotine showed a similar effect.

Yılmaz et al. (3) evaluated the skeletal system by double staining method in the study of the protection of melatonin against the teratogenic effect of nicotine on embryonic bone development. Similar to our study, they applied nicotine as 3 mg-6 mg/kg by s.c. In parallel with the dose of nicotine, they found statistically significant reductions in both bone length and ossification length of the bones in the front and hind extremities. In the study, the fetuses in the control group (sacrificed on the 20th day) were reported with a height of 29.57±1.25 mm and a weight of 2.40±0.13 g. These data were similar to the control group in our study as height (29.42±1.23 mm) and weight (2.36±0.15 g). While the height was 27.71±1.28 mm and the weight was 2.20±0.13 g in a low dose of nicotine, the height was 24.87±1.6 mm, and the weight was 1.99 ±0.15 g in a high dose of nicotine. The length and weight of the nicotine groups were close to the data in our study. Yılmaz et al. (3) have shown that the teratogenic effect of nicotine causes a statistically significant reduction in rat offspring's weight, height and bone development.

As a result of the literature review, several studies have shown that the effects of nicotine on the skeletal system and the effects of vitamin E on these damages are given. However, there were no studies conducted on nicotine and vitamin E related to the double skeletal staining method. In our study, we aimed to investigate the preservation of vitamin E against the negative effects of nicotine on bone and cartilage by double skeletal staining. In parallel with the dose increase, we detected that total bone/ossification increased in length, decreased ossification surface area and negatively affected bone development.

When the bones in the front and hind extremities (humerus, radius, ulna, femur, fibula, tibia) were compared with the nicotine and treatment groups, significant

increases were observed in ossification length, total bone length, ossification surface area in vitamin E groups. As a result, it was observed that nicotine negatively affected ossification in the embryonic period and thus decreased ossification percentages. It was found that vitamin E decreased the damage caused by nicotine and brought it closer to the values of the control groups. We conclude that supplementation of antioxidant vitamin E in women who continue to use nicotine during pregnancy will affect the skeletal development of the fetus positively. We think that this study will serve as an example and contribute to future studies.

CONCLUSION

It was concluded that exposure to nicotine during pregnancy delayed skeletal ossification and that vitamin E, which is an antioxidant, may be protective against the teratogenic effect of nicotine on the skeletal system.

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