The Effect of Curcumin on the Newborn Bone Development in Pregnant Rats that are Given Nicotine

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ABSTRACT

Oxidative stress plays an important role in fetus development. In this study, the role of nicotine in skeletal system changes were investigated with double staining 6 mg/kg nicotine; and the protective role of 50 and 100 mg/kg curcumin was also investigated. Pregnant rats were divided into 6 groups with 5 rats in each. Nicotine was applied to the experimental groups and curcumin was applied to the treatment groups in addition to nicotine. The upper and lower extremities of the offspring were examined under a stereomicroscope ossification rates were calculated by using the ImageJ Program. Tumor Necrosis Factor alpha (TNF - α), Interleukin 1ß (IL - 1ß), Interleukin 6 (IL - 6) levels were measured in tissue by using Enzyme-Linked ImmunoSorbent Assay method. Superoxide Dismutase (SOD), Glutathione (GSH), Glutathione disulfide (GSSG) Total Oxidant Status (TOS), Total Antioxidant Status (TAS), Calcium (Ca) and vitamin D (Vit D) measured by spectrophotometric assay. Oxidative Stress Index (OSI) and Total glutathione (GSH/GSSG) are useful indicators of oxidative stress tissues and were calculated as TOS-to-TAS and GSH-to-GSSG ratio respectively in the groups treated with curcumin were approximate to control group (P < 0.05). IL - 1ß, IL - 6 and TNF-α values were notably higher in Nicotine group than in all the other groups. (P < 0.05).

Abbreviations: TNF alpha: Tumor Necrosis Factor Alpha; SOD: Superoxide Dismutase; GSH: Glutathione; GSSG: Glutathione Disulfide; TOS: Total Oxidant Status; TAS: Total Antioxidant Status; Ca: Calcium; Vit D: vitamin D; OSI: Oxidative Stress Index; LDC: Low Dose Curcumin; HDC: High Dose Curcumin; ROS: Reactive Oxygen Species

Introduction

Smoking is now an important public health problem in all countries and is one of the reasons for preventable deaths in many countries. Around 7 million people die due to smoking every year in the world [1]. The increase in female users causes more problems especially during pregnancy [2,3]. Smoking during pregnancy can cause low birth weight, respiratory diseases and cancer sensitivity in infants [4]. Nicotine is the most important toxic component of cigarette in offsprings. That inhibits the activity of osteoblasts increases osteoclast differentiation from osteocytes and reducing the mechanical strength of bone. Low birth weight and skeletal development deformations in rat pups due to nicotine use during pregnancy and lactation have been reported [5,6]. Various agents
are used to prevent these teratogenic effects of nicotine. The most frequently studied agents are natural antioxidants [7]. Previous studies have shown that curcumin is a powerful antioxidant and is used in some cancer studies because of its antioxidant properties and also supports bone development [8-10]. Today, various experimental studies are carried out to determine the effects of harmful substances on bone development; and one of these studies is the Double Skeleton Staining Method. The process of staining bone and cartilage structures with hydyes is called Double Skeleton Staining [7].

Studies in which double skeletal staining has been used are grouped into two as Teratogenic Studies and Developmental Studies. Teratogenic effects, skeletal system effects and morphological studies on fetuses have been carried out. The dual skeletal staining method on the skeletal system is frequently used [7,11]. Oxidative stress is a principal parameter for evaluating nicotine effect on rat bone [12]. Nicotine at high doses to cause malformation loss and loss of bone mechanical strength in intact rats. The results of the previous studies showed that nicotine is a risk factor for osteoporosis [13,14]. According to previous studies, curcumin has been shown to have inhibitory and delaying effects on the osteoporosis process. Studies have shown that patients with significant bone injury after 6 months in curcumin-treated groups have decreased osteoporosis progression and bone turnover markers [15]. Nicotine is a harmful chemical which injures bone at development stage. In this study hypothesized that curcumin could play critical role on normalizing oxidative and inflammatory parameters which upregulated by nicotine. Therefore, we aimed to evaluate the effect of Low Dose Curcumin (LDC) and High Dose Curcumin (HDC) on humerus bone tissue after investigated with nicotine.

Material and Methods

The present study was conducted in accordance with the decision of Local Ethics Committee of Animal Experiments, Erciyes University dated 15/11/2017 with the number 17/106. 30 Wistar - Albino rats weighing 220-240 g were obtained from Erciyes University Experimental Animal and Clinical Research Center. Rats were mated and vaginal smear test was performed. Female rats with sperm on the smear test were considered as 0.5 days pregnant. During the experiment rats were kept at a constant temperature of 19 - 21°C and in a 12 hour light / dark environment. Experimental groups were formed through dividing pregnant rats into 6 groups. Invasive processes (i.p,s.c) were applied to the rats for 20 days during the pregnancy; and the gestational period lasted 21 days in average (Table 1).

**Table 1:** Creation of Experiment Groups.

| Experiment Groups | Injection Days (Every Day on Gestation Days) | Type of Injection |
|-------------------|---------------------------------------------|-------------------|
| Control           | 20-Jan                                      | i.p              |
| Nicotine (6mg/kg) | 20-Jan                                      | s.c              |
| Low Dose (50 mg/kg) Curcumin+Nicotine | 20-Jan                                      | i.p+s.c          |
| High Dose (100mg/kg) Curcumin+Nicotine | 20-Jan                                      | i.p+s.c          |
| Low Dose (50 mg/kg) Curcumin | 20-Jan                                      | i.p              |
| High Dose (100mg/kg) Curcumin | 20-Jan                                      | i.p              |

Dissolution and Sterilization of Curcumin and Nicotine

Nicotine and Curcumin were obtained from Sigma-Aldrich. PBS (Phosphate Buffer Saline) was used as a solvent for nicotine. Curcumin was dissolved in different volumes to provide the desired concentrations for each experimental group with DMSO (dimethyl sulfoxide) and PBS buffer. The DMSO ratio was adjusted to 0.05% of the total solution, the resulting solution was diluted with PBS; and the solutions prepared daily were sterilized by filtration.

Manipulation of Offspring

The offspring which were born were taken under Ketamine (75 mg / kg) + Xylazine (10 mg / kg) anesthesia. After cleaning the abdomen regions of the offspring with 70% alcohol, the abdominal walls were removed with a transverse incision, internal organs were removed, and the offspring were subjected to the Double Skeletal Staining Method (Table 2). For morphometric measurements, the images of the offspring were taken with Nikon E5700 camera via a stereomicroscope and were transferred to the computer. The length and area measurements of the bones in the images were measured using the Image J Program.

**Table 2:** Double staining method in neonatal bone.

| Technical Stages     | Solutions                           | Time  |
|----------------------|-------------------------------------|-------|
| Fixation             | 70% ethyl alcohol                   | 4-7 days |
| Degreasing           | Pure acetone                        | 1-3 days |
| **Double Staining**  |                                     | 7 days |
| 1st solution: 300mg Alcian Blue + 100ml 70% ethyl alcohol | 38-40 °C incubated |
| 2nd solution: 100mg Alizarin Red S + 100ml 95% ethyl alcohol | |
| 3rd solution: 1st solution + 2nd Solution + 100ml Glacial acetic acid | |
| 4th solution: Prepared by adding 1700 ml of 70% ethyl alcohol to the first three solutions | |
Biochemical Analysis

Tissue Preparation and Protein Quantification: Humerus used for analysis and placed into microcentrifuge tubes than washed 3x with 1 mL 100 mM PBS and aspirated. Stainless steel beads (1.6 mm blend) used for homogenization with 100 mM PBS. After homogenization, homogenates were centrifuged at 10,000 RPM for 30 minutes at +4°C. Supernatant was used as protein samples. Protein content was assayed using the BioRAD DC Protein Assay (BioRAD, 5000116).

Oxidative Stress Parameters: Oxidative stress parameters including TAS, TOS and SOD levels were determined by spectrophotometry. Tissue TAS level was determined by Erel, 2004 An antioxidant with a known concentration (1.65 mmol/l) was used as the standard to calculate antioxidant levels in the samples. The TAS level was expressed as mmol Trolox equivalent/l [mmol Trolox equiv./l] [16]. The tissue TOS level was measured by Erel, 2005 The assay was calibrated with a standard hydrogen peroxide solution (39.16 µmol/l). Results were expressed as µmol H₂O₂ equivalent/l. Other oxidative stress parameters such as GSH (Cat. No: EIA-GSHC, Thermo Fisher, USA), GSSG (Cat. No: a703002, Cayman, USA) and SOD (Cat. No: E-BC - K020, Elabscience, USA) performed by using spectrophotometer (Multiskan, Thermo Fisher) according to manufacturer’s instructions. Vitamin D analysis was determined using the commercial ELISA kit (EIA 539 DRG, Germany).

Inflammation Parameters and Ca²⁺

Protein samples were thawed, and commercial ELISA kits were used for the quantitative measurement of TNF - α (Cat. No: E - EL - R0019, Elabscience USA), IL-1β (Cat. No: E-EL-R0012, Elabscience USA), IL - 6 (Cat. No: E-EL-R0015, Elabscience USA) levels, according to the manufacturer’s instructions. Results are expressed in milligram per milliliter of proteins. ELISA was performed with protein samples extracted from tissues, according to the manufacturer’s instructions. Total Ca²⁺ concentration was evaluated by using calcium colorimetric assay kit (ab102505; Abcam) according to the manufacturer’s datasheet. 25 µL of standard solution and 25 µL of supernatant extracted from tissue, diluted 1 : 10, were mixed with 45 µL of chromogenic reagent and 30 µL assay buffer. The mixture was incubated at room temperature for 15 minutes in the dark. The signal was screened at 575 nm (Thermo Varioscan). The concentration of calcium in the samples was calculated according to [18].

Statistical Analysis

All analyses were conducted using SPSS version 23.0 (IBM Co., NY, USA). Data were presented as the mean ± standard deviation. For analysis of the differences in continuous variables among the groups, data were analyzed using one-way analysis of variance (ANOVA) followed by the post hoc Tukey’s test for parametric data and the Kruskal – Wallis test followed by the post hoc Dunn’s test for nonparametric data. Statistical significance was defined as a two-tailed P < 0.05.

Results

Effects on Upper Extremity Long Bones

In study, long bones (humerus, radius, ulna) of the upper extremities were evaluated. Generally when 6 mg/kg nicotine was given, there was a statistically significant decrease in the region lengths and ossification percentages, which showed the ossification in these bones (P < 0.05) When the additional curcumin was given, it was determined that the ossification increased and approached to that of the Control Group (Figure 1), (Table 3).

Figure 1: Images of the upper extremity bones: (A) Control group; (B) Low-dose (50mg/kg) Curcumin group; (C) High-dose (100 mg/kg) Curcumin group; (D) Nicotine+ Low-dose Curcumin; (E) Nicotine+ High-dose Curcumin group; (F) Nicotine group
Table 3: Upper extremity long bones (Ossification rate).

|       | Humerus |          |          | Radius |          |          | Ulna |          |          |
|-------|---------|----------|----------|--------|----------|----------|------|----------|----------|
|       | 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 | 6.95 | 3.6 | 52.17±3.24 | 5.09 | 3.23 | 61.49±5.14 | 6.17 | 4.16 | 61.59±5.38 |
| Nicotine | 30 | 6.79 | 3.07 | 43.99±2.93ab,c,d | 4.94 | 3.04 | 51.86±4.83ab,c,d | 5.99 | 3.01 | 52.63±3.91ab,c,d |
| N+LDC | 30 | 6.92 | 3.18 | 47.91±3.03ab,c,d | 4.97 | 3.09 | 54.28±4.82ab,c,d | 6.1 | 3.37 | 59.87±4.06ab,c,d |
| N+HDC | 30 | 6.91 | 3.66 | 52.04±4.44 | 5.05 | 3.24 | 60.85±4.28 | 6.09 | 4.19 | 61.49±5.81 |
| LDC | 30 | 6.89 | 3.64 | 52.87±3.19 | 5.11 | 3.21 | 61.12±4.06 | 6.22 | 4.14 | 62.50±4.86 |
| HDC | 30 | 6.95 | 3.69 | 53.17±4.73 | 5.14 | 3.25 | 61.27±4.37 | 6.29 | 4.21 | 64.35±5.64 |

Note: ANOVA test P<0.05 was considered statistically significant; N:Nicotine; HDC: high dose curcumin; LDC: low dose curcumin.

Effects on Lower Extremity Long Bones

In study, long bones of the lower extremity (femur, tibia and fibula) were also evaluated. A statistically significant decrease was found in the percentage of ossification in only the nicotine-treated group (P < 0.05). It was determined that ossification was more likely to reach the values of the Control Group in additional curcumin to nicotine and curcumin groups (Figure 2), (Table 4). In our study, the average offspring size was lower in the groups to which nicotine was administered compared to the control group and to the group to which curcumin was administered. There were dead births in the groups to which nicotine was administered; and it was determined that maternal food consumption decreased in the nicotine-administered group (Table 5).

Table 4: Lower extremity long bones (Ossification rate).

|       | 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 | 6.32 | 3.09 | 49.84±3.23 | 5.93 | 3.13 | 49.66±3.56 | 5.62 | 3.23 | 44.3±4.84 |
| Nicotine | 30 | 6.08 | 2.47 | 40.73±4.18ab,c,d | 5.65 | 2.27 | 39.13±3.52ab,c,d | 5.2 | 1.83 | 34.96±3.33ab,c,d |
| N+LDC | 30 | 6.13 | 3 | 43.14±4.43ab,c,d | 5.77 | 2.73 | 44.08±4.09ab,c,d | 5.6 | 2.29 | 39.77±4.29ab,c,d |
| N+HDC | 30 | 6.26 | 3.04 | 48.51±4.87 | 5.89 | 3.07 | 48.58±4.08 | 5.63 | 3.19 | 44.7±4.34 |
| 50 LDC | 30 | 6.29 | 3.14 | 48.6±4.26 | 5.93 | 3.14 | 49.89±4.62 | 5.67 | 3.24 | 46.67±4.22 |
| 100 HDC | 30 | 6.23 | 3.1 | 47.06±3.28 | 5.59 | 3.1 | 49.73±4.70 | 5.65 | 3.21 | 46.09±3.84 |

Note: ANOVA test P<0.05 was considered statistically significant; N:Nicotine; HDC: high dose curcumin; LDC: low dose curcumin.

- a) It is significant when compared with the control group.
- b) It is significant when compared with the HDC group.
- c) It is significant when compared with the LDC group.
- d) It is significant when compared with the N+HDC group.
- e) It is significant when compared with the N+LDC group.

Figure 2: Images of the lower extremity bones: (A) Control group. (B) Low-dose (50mg/kg) Curcumin group. (C) High-dose (100 mg/kg) Curcumin group. (D) Nicotine+ Low-dose Curcumin group. (E) Nicotine+ High-dose Curcumin group. (F) Nicotine group.
Table 5: Maternal mortality and food consumption rates during pregnancy. Offspring macroscopic data.

|                        | Control | Nicotine 6mg/kg | N+LDC 50mg/kg | N+LDC 100mg/kg | Curcumin 50mg/kg | Curcumin 100mg/kg |
|------------------------|---------|-----------------|---------------|----------------|------------------|-------------------|
| Gestation food consumption (g/animal/day) | 15      | 13.3            | 13.9          | 14.2           | 14.2             | 14.7             |
| Number of live fetuses for each maternal | 12±3.2  | 11 ± 2.6        | 11 ± 3.2      | 11 ± 3.3       | 12 ± 1.7         | 12 ± 3.4         |
| Maternal mortality     | 0       | 1               | 1             | 0              | 0                | 0                |

Macroscopic malformations in newborn

|                        | Soft tissue | Skeletal | Rudimentary tail |
|------------------------|-------------|----------|------------------|
| Control                | 0           | 0        | 0                |
| Nicotine 6mg/kg        | 3           | 1        | 1                |
| N+LDC 50mg/kg          | 2           | 0        | 0                |
| N+LDC 100mg/kg         | 0           | 1        | 0                |
| Curcumin 50mg/kg       | 0           | 1        | 0                |
| Curcumin 100mg/kg      | 0           | 0        | 0                |

Biochemical Assay Results (Assessment of Oxidative Stress Parameters)

The highest TAS value obtained at Control group whereas the lowest TOS value obtained at Nicotine group. Curcumin administration increased antioxidant status significantly in a dose dependent manner when compared with Nicotine group. Dose dependent curcumin showed more antioxidant effect while used without nicotine (Figure 3). TOS values showed that the highest oxidant effect observed at Nicotine group. Control group has the lowest value (Figure 4). Oxidative stress index was calculated as ratio of TOS and TAS. OSI in Nicotine group considerably higher than the other groups (Figure 3). OSI decreased in curcumin administrated groups. In Nicotine group SOD has highest value compared to the other groups. Dose dependent curcumin with nicotine has lower value than curcumin without nicotine. Vitamin D showed inversed effect while comparing with SOD. In Control group Vitamin D has highest value than the other groups (Figure 4). GSH and GSH/GSSG has highest value in control group whereas GSSG has highest value in nicotine group. Curcumin increased GSH and GSH/GSSG values while decreased GSSG values comparing nicotine and control groups respectively (Figure 5). According to these results curcumin markedly decreased oxidative stress via stimulating an antioxidant system.

Figure 3: Mean TAS, TOS and OSI levels in all groups. The data are expressed as mean ± standard deviation (*P < 0.05 vs. Nicotine group. One way ANOVA, post hoc Tukey test).
Assessment of Inflammatory and Calcium Parameters

IL-1β, IL-6 and TNF-α values were notably higher in the Nicotine group than in all the other groups. Dose depended curcumin with nicotine groups has higher value than dose depended curcumin without nicotine (Figure 6). Curcumin particularly showed an anti-inflammatory effect against nicotine via reducing tissue levels of IL-1β, IL-6 and TNF-α. In Nicotine group intracellular calcium value has the highest level. Nicotine treatment following dose depended curcumin administration increased calcium level slightly whereas curcumin administrated groups without nicotine treatment have higher value (Figure 7).

Discussion

Nicotine easily passes through the placenta due to its lipophilic nature. Several researchers used different doses of nicotine in rats during pregnancy in their experimental studies. In the literature, these doses range from 1.67 mg/kg to 7.5 mg/kg. [7,19,20]. investigated the protective effects of melatonin against the effects of nicotine on bones in their study; and found that the percentage...
and rate of ossification was low in the upper and lower extremity bones of the group to which 6 mg/kg nicotine was administered (31.42 ± 10.33) compared to the control group (43.71 ± 2.33). In their study, they stated that this ratio was close to the Control Group in the melatonin group [7,21]. Investigated the negative effects of nicotine on bone development. In their study, they administered 3 mg/kg nicotine given i.p. during pregnancy and lactation. At the end of the experiment, it was reported that femur lengths of 21 days old offspring in the group treated with nicotine (19.1 ± 1.6 mm) were significantly lower than the control group (21.1 ± 0.3) and in their study they showed maternal nicotine exposure resulted in decreased birth weight, pregnancy weight gain, and bone lengthening, and increased apoptosis [21]. The lactation period of the mothers who smoked was measured for 24 hours in the study of [22], which were evaluated as 113 ± 179 ml in the Control Group and 47 ± 122 ml in the group in which mothers smoked [22,23]. injected 2 mg/kg nicotine s.c into pregnant rats in their studies; and determined the dose of 2 mg/kg nicotine as 12 cigarettes per day. In their study, they reported that total cholesterol values in mother serum were higher than controls [23,24]. studied the teratogenic effect of nicotine in female fetuses exposed to cigarette smoke for 14 days before pregnancy and for 20 days during pregnancy, and male rats that were exposed to cigarette smoke for 28 days before pregnancy. In rats exposed to 18.6 ± 2.07 mg / m³ Nicotine and 600 mg, 41.8 ± 3.71 mg / m³; a significant increase was reported in the number of missing or non-ossified bones in rats exposed to nicotine [24,25]. evaluated the osteoclast genesis and endochondral ossification of long bones of fetuses in pregnant rats exposed to nicotine. In their study, they used 2 mg/kg nicotine s.c for 20 days in pregnant rats.

As a result of their experiments, they reported a significant decrease in the length of femur in fetuses exposed to nicotine compared to control group [25]. There are many studies conducted on pregnant rats and individuals showing that nicotine causes a decrease in fetus ossification rate. In the literature, this effect has been reported to be reduced by means of various antioxidant agents [3]. One of these substances is curcumin. Curcumin is used as a colorant in spices, food and textiles, and in diets, as well as in many diseases [26]. Many different pharmacological activities and biological benefits of curcumin have attracted considerable attention in recent years [27]. Many studies have shown that curcumin has anti-oxidant, anti-carcinogen, anti-inflammatory, anti-allergic, anti-dementia effects and is a free radical scavenger [28-30]. gave 30 mg / kg curcumin to the rats treated with periodontitis and reported that alveolar ossification increased [28,29]. investigated the effect of curcumin on glucocorticoid-induced osteoporosis in rats and reported that curcumin increased osteoblast activity and decreased osteoclast activity [29,30]. applied 50, 100, 200 mg/kg curcumin as a gavage for 30 days to heal Benzo [a] pyrene-induced DNA damage in stomach tissues [31]. In the study, it was stated that curcumin reduced the negative effect of nicotine on neonatal skeletal development and values approached the control group.

Experimental studies on animals or cultured human cell lines support a role of polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis [32]. When bone fractures occur, a remarkably high yield of radicals is generated. It is suggested that as a break occurs, the minimal crystallites separate at grain boundaries with no major chemical changes, but the tightly bound collagen strands running through the mineral phase are forced to break homolethically. Some react with oxygen and yield oxygen radical metabolites [33]. Cigarette smoke is a complex mixture of more than 4700 chemical compounds including free radicals and oxidants. Toxicity exhibited by cigarette smoke may be due to combined action of these compounds inducing many cellular processes mediated through Reactive Oxygen Species (ROS). Major player probably nicotine as it is present in tobacco, in higher concentrations. Meanwhile, elevated levels of ROS can damage proteins, lipids, and DNA, eventually trigger oxidative stress and leading to cell death [34,35]. Oxidative damage to bio-macromolecule has been proved in the etiology of a wide variety of acute and chronic diseases, including osteoporosis [36].

Measurement of TOS, TAS, Vitamin D and SOD is a crucial biomarker to evaluate oxidative damage [37-41]. In this study we detected that curcumin increased the TAS level whereas decreased the TOS levels both nicotine threatened groups and nicotine untreated groups. The highest value of SOD was observed in Nicotine group and as well as the lowest value was recorded at control group. Nicotine significantly increased SOD level by stimulating reactive oxygen system thus as an antioxidant curcumin slightly decrease the effect of nicotine and approximate the values to control group. Vitamin D is an antioxidant ant it decreases when cell damaged [42]. In Nicotine group Vitamin D has the lowest value and Control group has the highest value. After nicotine treatment due to the ROS activity Vitamin D level significantly decreased but after dose dependent curcumin treatment it increased slightly.

GSH, present in concentrations of 2-10 mM within cells, is the primary determinant of the cellular redox environment and exists mainly as the biologically active reduced-thiol form [43]. The oxidation of GSH to GSSG and subsequent decrease in the GSH/GSSG ratio is often associated with oxidative stress. Thus, the GSH/GSSG ratio is a simple and useful indicator of cellular redox state [44,45]. In the current study GSH decreased by the effect of the nicotine while curcumin treatment values approximated to Control group. Likewise, GSH/GSSG showed same attitude but GSSG values were observed as just the opposite. Inflammatory responses observe after necrotic cell death. Nicotine induces necrosis following inflammation. Inflammatory markers have been widely used to show inflammation situation such as IL-6, IL-1β and TNF-α. The serum concentrations of those markers increase after nicotine
Intracellular calcium signaling controls scores basic cellular processes including proliferation, differentiation, and cellular motility [50]. Calcium levels are conserved at very low concentrations intracellularly via its removal to the extracellular environment and sequestration in the endoplasmic reticulum. As such, it is a powerful second messenger important in proliferation, differentiation, mitosis, and motility. In bone cells, an extracellular influx and intracellular release is rapidly activated by strain, pressure, and fluid flow [51-53]. In the current study Nicotine group has the lowest Calcium level. After nicotine treatment following curcumin administration calcium level slightly increased while dose dependent curcumin treatment groups has highest calcium level. Thus, we suggest that curcumin is effective in reducing oxidative stress and bone loss induced by nicotine in rats.

**Conclusion**

As a result of our study, it was determined that there was a decrease in bone development and decrease in ossification rate in fetuses of pregnant rats exposed to nicotine. Different doses of curcumin were given to the rats against nicotine, and it was determined that the number of non-ossified bones decreased, and decrease in bone development and decrease in ossification rate was markedly improved in the high-dose curcumin-treated groups. As a conclusion curcumin in therapy after nicotine administration markedly improved anatomical and biochemical findings and prohibited oxidative stress and inflammation. According to these data we propose that curcumin at the 100 and 150 mg may be used as a potential therapeutic agent to prevent bone loss induced by nicotine and our results will be beneficial in model studies which will be conducted on curcumin and nicotine.

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