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ORIGINAL ARTICLE

Effects of pseudoephedrine on rat fetal bone development: evaluation by three different methods

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Abstract

Pseudoephedrine (PSE) is an agent that is contained in common cold medications. The agent, which is used to treat cold and cough, is the fourth most prescribed drug group in some countries. During pregnancy, expectant mothers use PSE for colds and other reasons. One out of every four expectant mothers use PSE alone or in combination with other medicines for various reasons. This study was aimed to investigate effects of PSE on long bones development in rat during fetal growth. Pregnant rats were divided into five groups: control and four experimental groups (25 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg PSE). Between 1 and 20 days of pregnancy, PSE was given to them by gavage. Weights and heights of fetuses isolated by cesarean on the 21st day were measured. Ossification of femur and humerus was examined by three different methods mentioned earlier. Depending on the dose increase, all morphometric data, ossification rate and bone length of the fetuses were decreased. Besides, it was determined that the amount of Calcium in the bone tissue decreased in the analyzes made with SEM–EDX Analysis. The data obtained from this study reveal that the use of PSE during pregnancy disrupts the existing balance in the bone and negatively affects ossification due to the dose increase. In conclusion, we present descriptive and novel data on the effects of PSE use during pregnancy on the bone development of rat fetal long bones.

Keywords EDX analysis · Morphology · Ossification · SEM · Teratology

Introduction

Pseudoephedrine (PSE) is an agent that is included in the amphetamine class, which is the stereoisomer of ephedrine. It is usually prescribed for the treatment of cold and cough. Drugs containing PSE are the 4th most commonly prescribed drug group in the USA and usually prescribed for the treatment of cold and cough. Thus PSE is one of the most commonly used over-the-counter medications. Although the drug is used so frequently, it is not known what effects it has on the newborn as a result of maternal exposure. (Kaufman et al. 2002; Thorpe et al. 2013). Although there is insufficient evidence of a negative effect of PSE, the results of some studies suggest that it should be used with caution (Głowacka and Wiela-Hojeńska 2021). Its isomers, ephedrine, phenyl-propanol-amine

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and epinephrine has been reported to cause cardiac, ventral wall, aortic and leg anomalies on animal models and humans (Werler 2006; Saffari et al. 2019). It is also said to have teratogenic effects when used PSE with phenylephrine, and phenylpropanolamine (Gilbert-Barness and Drut 2000), but its effects on fetal bone development are not known if PSE is used alone.

The bone that constitutive the skeletal system begins to develop in the embryonic period (Dudek 2010). The first step in fetal bone formation is the differentiation of chondrocytes from mesenchymal cells. Although some skeletal parts, such as cranium and clavicula are formed by membranous ossification, especially extremity bones are formed by endochondral ossification. With the aggregation of mesenchymal cells, these cells differentiate into chondrocytes and form cartilage plaques. Although these cartilaginous plaques proliferate on the one hand, they also mature in the central regions and form hypertrophic areas. With the continuity of the process, primary ossification centers are formed. Thus, the hypertrophic place leaves the bone and ossification occurs (Nakamichi et al. 2020). Calcium (Ca) and Phosphorus (P) are essential elements of ossification. Other components, such as Magnesium (Mg), Potassium (K), Sodium (Na) in the bone are also important factors that determine the quality of the bone. During fetal bone development, sufficient presence of these elements in bone is essential for normal ossification. Therefore, the decrease or increase in the amount of these elements directly affects ossification and bone health (Sethi et al. 2020).

The use of this drug, which is one of the basic substances of almost all cold medicines, is increasing. Pregnancy use category of PSE has been determined as "C" according to the American Drug and Food Administration (FDA) (Callaghan 2000). However, it is not known whether pseudoephedrine will harm an unborn baby. This drug should not be taken during pregnancy unless the benefit outweighs the risk to the fetus. Although it is not recommended to take the drug for more than 7 days during pregnancy, since the drug is often included in other decongestants, it should be clearly stated what effects the drug has on long-term exposure. Therefore, possible negative effects that may occur in maternal use of the drug should be thoroughly investigated. Considering previous public health studies and warnings in the literature, we decided to investigate the effects of the drug on long bones. Our hypothesis was that this drug had adverse effects on neonatal bones and adversely affected ossification. To confirm this hypothesis, we analyzed bone and ossification ratios and lengths by the double staining skeleton staining method. We determined the differences between the groups by measuring the ossification stages, the diameters and lengths of the lacunae and chondrocytes with Scanning Electron Microscope (SEM). We analyzed the differences between the groups by detecting the minerals directly affecting ossification by SEM-energy-dispersive X-ray (EDX) analysis (Scimeca et al. 2018).

Materials and method

This study was approved by Erciyes University Animal Experiments Local Ethics Committee's decision dated 17/11/2017 and numbered 17/104. The care, feeding, drug applications to the animals used in the study and the sacrification of the animals at the end of the experiment were carried out within the Experimental Research Application and Research Center (DEKAM). All the procedures performed during the study were carried out according to the rules specified in the ethics committee directive. This work was supported by Erciyes University Scientific Research Projects Unit with the project code TYL-2018–7924. Within the scope of the study, 20 Sprague Dawley (SD) female rats (approximately 7–9 weeks of age) were obtained from DEKAM. The female rats to be used in the study were kept separately from the male rats for 20 days. Experimental rooms were environmentally controlled with 12-h light/12-h dark cycles, and fresh air changes per hour. Room temperature was maintained at 20.0–26.0 °C (30.0–70.0% relative humidity) for rats. The rats, which were found not to be pregnant, were placed in the same cage at 17:00 with male rats (1:1 basis) to conceive. The rats were kept in the same cage until 08:00 the next day. At the end of the process, vaginal smear samples taken from female rats were examined under a microscope. Females with spermium in the vaginal smear were considered being pregnant for 0.5 days.

Drug applications

Doses to be given to experimental animals (25 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg PSE) were obtained from the equations used by the FDA and other researchers. It was calculated using the equations in literature (Human Equivalent Dose (mg/kg) = Animal Dose (mg/kg) × K_m ratio) (Rockville, Food, and Administration 2005; Nair and Jacob 2016). The amount of 25 mg/kg PSE used in our study is equivalent to the safe maximum human equivalent dose (240 mg/day) that can be taken daily in humans. PSE is sold on the market alone or in combination with another medicine with different trade names. Commercially available drug—Sudafed line—containing only PSE as an active agent and containing lactose and povidone, which is known to have no adverse effects in trace amounts was purchased. PSE was dissolved in 1 ml SF (saline) and given to pregnant rats by gavage every day between 16:30 and 17:00. Before gavage of the rats, the measurement was taken from the mouth level of the rat to the sternum to determine the length of the gavage needle. In order to perform gavage properly and to prevent

aspiration, applications were made in accordance with the general guidelines. After the day of SF and PSE administration (at the end of the 20th day) in both the control group and the experimental groups, pregnant rats were anaesthetized using ketamine (75 mg/kg) and xylazine (10 mg/kg). The abdomen of the pregnant rats who were anaesthetized was cleaned with 70% alcohol. Then, a transverse incision was made with the help of a scalpel, and the anterior wall of the abdomen was opened. Fetuses in the uterus were dissected together with their placenta. As soon as the isolation of the fetuses from the mother rats was over, the mother rats were sacrificed by cervical dislocation.

Double skeleton staining method

Fetuses were skinned, macerated, and stained with Alcian Blue (cartilage, Sigma-Aldrich) and Alizarin Red (ossified bones, Sigma-Aldrich). The detailed protocol was previously described was applied to the fetuses obtained (Schneider 2013).

Acquisition of images

Femur and humerus belonging to the stained fetuses were placed on a millimeter paper and placed on a stereomicroscope. Bones were photographed with the Nikon 500 camera. The obtained images were transferred to the computer environment and measurements were made on bone and cartilage areas by ImageJ software.

SEM and EDX analysis method

Femur and humerus belonging to fetuses were properly insulated and kept in glutaraldehyde solution with 0,1 Molar phosphate buffer in a pH range of 7.2–7.4 for 24–48 h. Bones were kept in 1% detergent (sodium dodecyl sulfate) for 48 h at 37 °C to remove soft tissues. The bones, whose soft tissues were removed, were washed 3 times with 5 min of distilled water. Immediately afterwards, it was passed through the increasing acetone series (%50–70–80–90–100–100–100) for 15 min and passed in order. After the acetone series, bones were kept in ether (24 h) and degreased. Bone tissues taken in petri dishes were kept at room temperature for 48 h and dried. The dried bone tissues were covered with gold and palladium with the sputter coater device for examination in ZEISS Gemini 500-71-08 branded SEM device. Besides EDX analysis was performed in the epiphyseal and diaphyseal regions of the bones. The images obtained with SEM are transferred to the computer environment; lacuna length, lacuna width, chondrocyte length and chondrocyte width were measured by ImageJ software. Calibration adjustment was made between the original image and the photo image to give the correct result in both double staining skeleton and SEM method. As a result of the calibration, the distance to be measured was determined manually with the wand tool feature of the ImageJ program and the results were obtained. In addition, on the images obtained by SEM analysis; the area between the interfibrils was calculated using the binary-threshold feature of ImageJ software (Pazzaglia et al. 2016).

Statistical analysis

D'Agostino Pearson's omnibus test was used to identify the normal distribution of the data. In the case of normal distribution, quantitative variables were compared using an one-way analysis of variance (ANOVA) and Tukey's post hoc test. Kruskal–Wallis test and Tukey's post hoc test were used for comparing the quantitative with the abnormal distribution. The data were expressed as the mean of normalized data ± standard deviation of the mean. $p < 0.05$ was considered as statistically significant.

Results

Morphometric and double staining method findings

The length and weight data and statistical analysis of fetuses are shown in Table 1. Placental weights of the 200 mg/kg PSE group were significantly different from the control group ($p < 0.05$). Biparietal length, occipitofrontal length measurements were different between the control group and all experimental groups ($p < 0.05$). In head-aft length, there was no significant difference between the control group and the 25 mg/kg PSE group, but there was a significant difference between them and the other groups ($p < 0.05$). In addition to morphometric differences, a decrease was observed in the number of fetuses due to dose increase. It was observed that the humeral length and ossification length decreased depending on the dose increase, and there was a significant difference between the control group and all of the experimental groups ($p < 0.05$). When the total bone area of the humerus is compared; There was a significant difference between control and 50 mg/kg, 100 mg/kg, 200 mg/kg PSE groups ($p < 0.05$). As a result of the dose increase, the length and ossification rates in the humerus could also be seen macroscopically (Table2, Fig. 1A). The length and area data of the bone and ossification of the femur are shown in Table 3. The femur lengths and ossification lengths of the pups in the control group were statistically significantly different from all the pups in the experimental group ($p < 0.05$). The total bone area of the femur and ossified area were severely different between the high dose group and the other groups ($p < 0.05$). This significant difference

Table 1 Comparison of various weights and lengths for fetuses

Groups	*nf*	WF (g) (mean ± SD)	PW (g) (mean ± SD)	BPD (cm) (mean ± SD)	OFD (cm) (mean ± SD)	CRL (cm) mean ± SD
Control	45	1.96 ± 0.10^{a}	0.59 ± 0.07^{a}	0.72 ± 0.06^{a}	1.29 ± 0.09^{a}	2.72 ± 0.12^{a}
25 mg/kg pse	44	1.95 ± 0.12^{a}	0.58 ± 0.14^{ab}	0.68 ± 0.06^{b}	1.18 ± 0.12^{b}	2.67 ± 0.16^{a}
50 mg/kg pse	37	1.94 ± 0.09^{ac}	0.57 ± 0.05^{ab}	0.63 ± 0.07^{c}	1.06 ± 0.09^{c}	2.51 ± 0.12^{bc}
100 mg/kg pse	36	1.89 ± 0.09^{bc}	0.54 ± 0.06^{ab}	0.59 ± 0.05^{c}	0.94 ± 0.07^{d}	2.46 ± 0.15^{c}
200 mg/kg pse	32	1.82 ± 0.08^{b}	0.51 ± 0.05^{b}	0.60 ± 0.07^{c}	$0,89 \pm 0.10^{d}$	2.50 ± 0.09^{bc}
p		< 0.001	0.010	< 0.001	< 0.001	< 0.001

pse pseudoephedrine, *nf* number of fetuses, *WF* weight of fetus, *PW* placenta weight, *BPD* biparietal diameter, *OFD* occipitofrontal diameter, *CRL* Crown–Rump length

*An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

**Data were expressed as mean ± standard deviation. The same letters in the same column represent the similarity between the groups and the different letters represent the difference between the groups

Table 2 Comparison of lengths and areas of humerus

Groups	nb	TBL (mm) (mean ± SD)	TOL (mm) (mean ± SD)	TBA (mm^2) (mean ± SD)	TOA (mm^2) (mean ± SD)
Control	27	4.05 ± 0.18^{a}	1.69 ± 0.18^{a}	3.20 ± 0.29^{a}	1.24 ± 0.12^{a}
25 mg/kg pse	27	3.88 ± 0.20^{b}	1.51 ± 0.05^{b}	3.05 ± 0.17^{ab}	1.15 ± 0.03^{b}
50 mg/kg pse	27	3.76 ± 0.34^{bc}	1.49 ± 0.03^{bc}	2.89 ± 0.32^{b}	1.13 ± 0.05^{b}
100 mg/kg pse	27	3.74 ± 0.07^{bc}	1.45 ± 0.02^{bc}	2.88 ± 0.49^{b}	1.09 ± 0.09^{b}
200 mg/kg pse	27	3.72 ± 0.04^{c}	1.44 ± 0.07^{c}	2.26 ± 0.19^{c}	$0,87 \pm 0.08^{c}$
p		< 0.001	< 0.001	< 0.001	< 0.001

pse pseudoephedrine, *nb* number of bones, *TBL* total bone length, *TOL* total ossification length, *TBA* total bone area, *TOA* total ossification area

*An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

**Data were expressed as mean ± standard deviation. The same letters in the same column represent the similarity between the groups and the different letters represent the difference between the groups

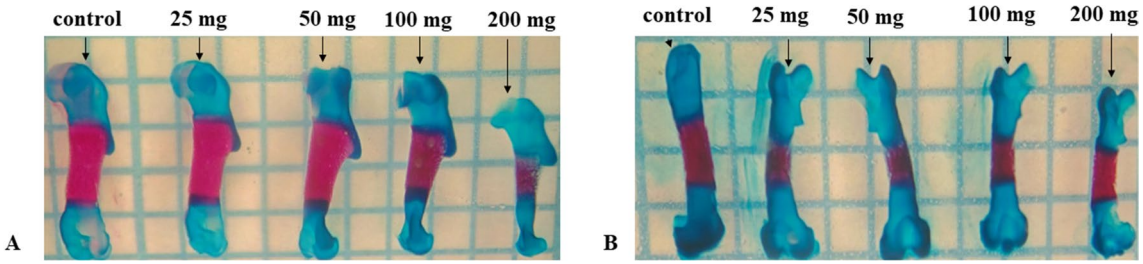

Fig. 1 **A** Changes in the humerus due to dose increase. **B** Changes in the femur due to dose increase (black arrows indicate doses)

can be easily distinguished macroscopically on millimetric paper (Fig. 1B).

SEM–EDX findings

The concentrations of Ca, P, and C were analyzed by SEM–EDX point analysis. The Ca/P ratio in 200 mg/kg PSE ($p = 0.00$), 100 mg/kg PSE ($p = 0.03$), 50 mg/kg PSE ($p = 0.05$) was significantly lower than in control group whereas there was no significant difference between the ratio in control and 25 mg/kg PSE groups. The C/Ca ratio in 200 mg/kg PSE ($p = 0.01$), 100 mg/kg PSE ($p = 0.00$) was significantly higher than in control group whereas there was no significant difference between the ratio in control, and 25 mg/kg PSE, and 50 mg/kg PSE groups. When considering the Ca/P ratio, although there was a decrease in amount, it was determined that there was a significant decrease only between the control group and the 200 mg/kg experimental group. No significant difference was observed between the other groups. The amount of calcium was evaluated between

Table 3 Comparison of lengths and areas of femur

Groups	nb	TBL (mm) (mean ± SD)	TOL (mm) (mean ± SD)	TBA (mm^2) (mean ± SD)	TOA (mm^2) (mean ± SD)
Control	27	3.29 ± 0.33^{a}	1.27 ± 0.21^{a}	2.17 ± 0.16^{a}	0.62 ± 0.14^{a}
25 mg/kg pse	27	3.22 ± 0.24^{ab}	1.10 ± 0.22^{b}	2.02 ± 0.31^{ab}	0.62 ± 0.08^{a}
50 mg/kg pse	27	3.11 ± 0.32^{ab}	1.02 ± 0.12^{bc}	2.03 ± 0.13^{ab}	0.62 ± 0.03^{a}
100 mg/kg pse	27	3.11 ± 0.21^{ab}	0.97 ± 0.19^{c}	1.97 ± 0.34^{b}	0.55 ± 0.10^{b}
200 mg/kg pse	27	3.03 ± 0.31^{b}	0.92 ± 0.14^{c}	1.78 ± 0.29^{c}	0.49 ± 0.08^{c}
p		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

pse pseudoephedrine, *nb* number of bones, *TBL* total bone length, *TOL* total ossification length, *TBA* total bone area, *TOA* total ossification area

*An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

**Data were expressed as mean ± standard deviation. The same letters in the same column represent the similarity between the groups and the different letters represent the difference between the groups

the groups, a statistically significant difference was found between the control group and the 25 mg/kg, 100 mg/kg and 200 mg/kg PSE groups ($p < 0.05$) (Fig. 2).

SEM findings

With ImageJ software on SEM images, 4 different data were obtained, including the length of 10 lacunae belonging to each group, the width of the lacuna, the length of the chondrocyte in the lacuna and the width of the chondrocyte. Statistical analysis of the data can be seen in Fig. 3 and Table 4. According to the data, it was determined that the lengths of lacuna belonging to the control group had a statistically significant difference from all experimental groups ($p < 0.05$) (Fig. 3A).

When the average widths of the lacunae were examined, statistically significant difference was found between the control group and all experimental groups ($p < 0.05$) (Fig. 3B). When the average lengths of chondrocytes were examined, it was found that there was a statistically significant difference between the control group and the experimental groups between 100 mg/kg PSE and 200 mg/kg PSE groups ($p < 0.05$) (Fig. 3C). Depending on the increase in dosage, the average length of the lengths of chondrocytes was noticeable but was not statistically significant. When the average widths of chondrocytes were examined, statistically significant difference was found between the control group and all experimental groups ($p < 0.05$) (Fig. 3D).

Endochondral ossification centers can be selected in the epiphyseal regions of SEM images of bones obtained from both control and experimental groups (Fig. 4A). Resting, proliferation, hypertrophy, calcification and ossification regions had similar histological arrangements in all groups (Fig. 4B). When the ossification areas of the preparations belonging to the control group are examined, the presence of chondrocytes located in the lacunae in the resting area under the perichondrium is distinguished. In the proliferation zone adjacent to the resting zone, isogenic groups were present as

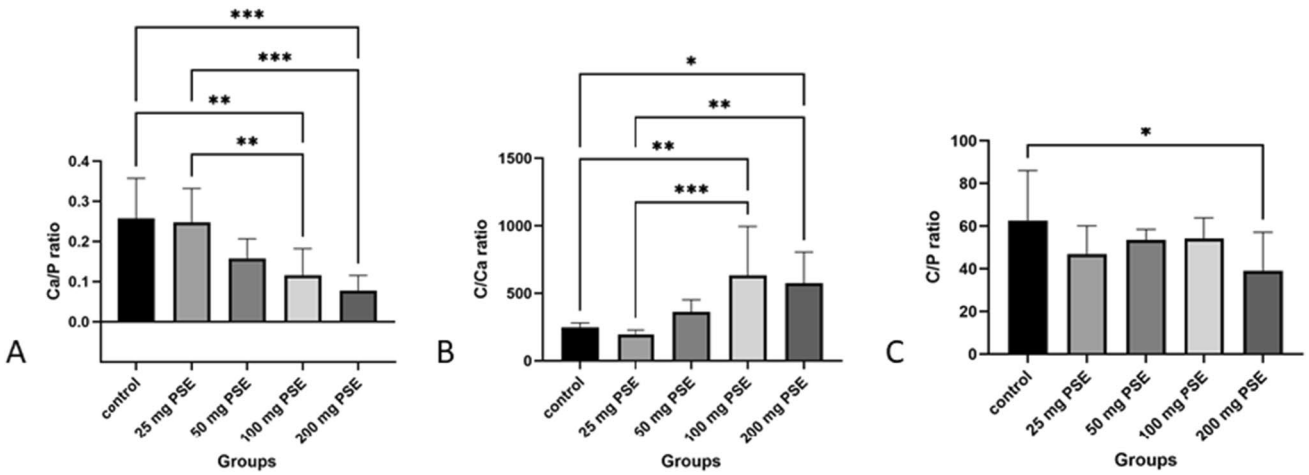

Fig. 2 **A** Ratio of Ca/P between groups. **B** Ratio of C/Ca between groups. **C** Ratio of C/P between groups. *PSE* Pseudoephedrine. *An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

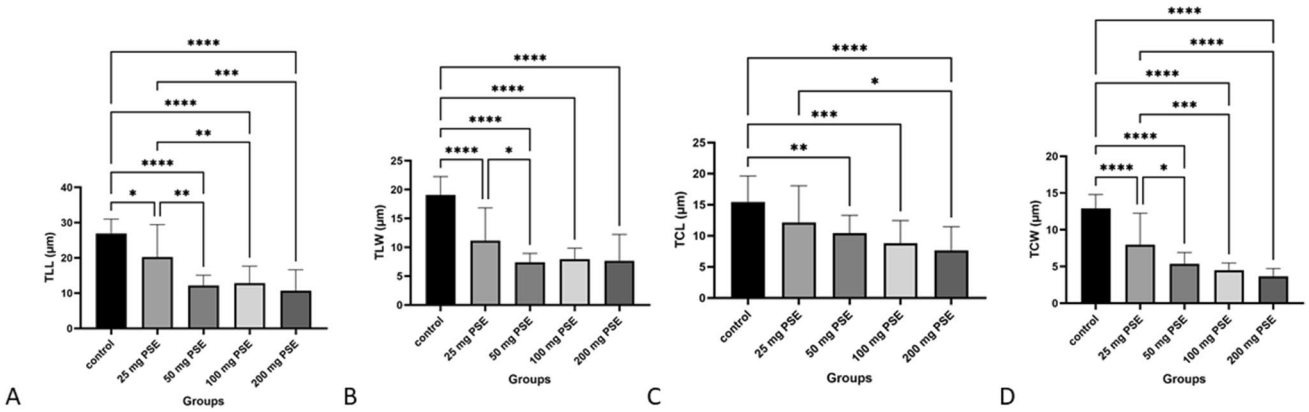

Fig. 3 **A** *TLL* total lacunae length **B** *TLW* total lacunae width **C** *TCL* total chondrocyte length **D** *TCW* total chondrocyte width. *An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

Table 4 Statistics of chondrocytes and lacunae data

Groups	*n*	LL (µm) (mean ± SD)	CL (µm) (mean ± SD)	LW (µm) (mean ± SD)	CW (µm) (mean ± SD)
Control	10	26.96 ± 4.38^{a}	15.17 ± 4.56^{a}	19.07 ± 3.57^{a}	12.97 ± 2.08^{a}
25 mg/kg pse	10	17.75 ± 8.92^{b}	11.82 ± 6.26^{ab}	11.83 ± 6.12^{b}	7.47 ± 4.25^{b}
50 mg/kg pse	10	12.81 ± 3.27^{bc}	10.06 ± 2.86^{ab}	7.42 ± 1.42^{b}	$5.29 \pm 1.39b^{c}$
100 mg/kg pse	10	12.83 ± 4.85^{bc}	8.80 ± 3.63^{b}	7.95 ± 1.91^{b}	4.50 ± 0.96^{c}
200 mg/kg pse	10	10.70 ± 5.93^{c}	7.77 ± 3.91^{b}	7.74 ± 4.72^{b}	3.73 ± 1.05^{c}
p		**< 0.001**	**0.010**	**< 0.001**	**< 0.001**

pse pseudoephedrine, *n* number of samples, *LL* lacunae length, *CL* chondrocyte length, *LW* lacunae width, *CW* chondrocyte width

*An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

**Data were expressed as mean ± standard deviation. The same letters in the same column represent the similarity between the groups and the different letters represent the difference between the groups

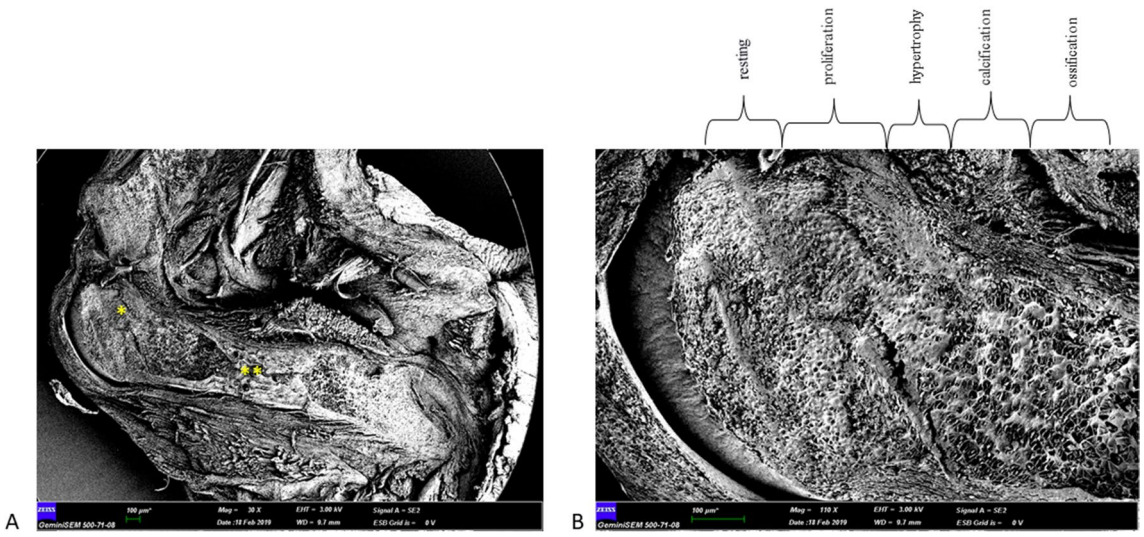

Fig. 4 **A** General view of epiphyseal (*) and diaphyseal region (**) **B** steps of the endochondral ossification

columns stacked on top of each other parallel to the long axis of the bone. The third step, the hypertrophy region, is distinguished by the presence of lacunae, which are enlarged and fused. The resorbed cartilage matrix between chondrocytes was observed as a thin septum. In the calcified cartilage area, the territory matrix was determined to acquire a calcified structure. Bone spicules were observed in the part of the ossification region, which is the last step of endochondral ossification, located closest to the diaphysis (Fig. 4B).

In all groups given PSE, as in the control group, chondrocytes, which were located in lacunae in the rest area, remained. In the group's given PSE, as in the control group, chondrocytes proliferating in the region were located in the vertical axis and parallel series. However, due to the increase in dose, the number of chondrocytes proliferating and the areas where the territory matrix was calcified were determined. At the same time, with the increase in dose, the cell bodies of the chondrocytes located in the lacunae in all experimental groups shrank (Fig. 5). Nevertheless, the apoptosis of chondrocytes and calcification of the territory matrix in the experimental groups in the calcification and ossification region was more severe than the control groups. When SEM images belonging to the experimental groups were examined, thinning was observed in collagen fibrils forming a lamellar bone or knitted bone structure (Fig. 6). There was also an increase in the empty space between interfibrils area (Fig. 7).

Discussion and conclusion

In a study by Smith et al., a single dose of 60 mg/kg PSE was administered to pregnant women in the last trimester of pregnancy, and no adverse effects were found on the fetal aorta, uterus and umbilical artery (Smith et al. 1990). However, this study is lacking in revealing the specific effects of this drug in long term, which is exposed during pregnancy, in the offspring. Some researchers have found that it has teratogenic effects when used with another drug. However, it has not been revealed what effect it has when used alone (Głowacka and Wiela-Hojeńska 2021; Werler et al. 1992). However, these studies are not experimental and observation-based, survey studies. Therefore, it causes confusion about the reliability of the studies. The lack of adequate experimental studies on a drug or an active agent, especially this drug, which is frequently used over the counter by pregnant women, is a major shortcoming. In order to avoid another thalidomide disaster, the effects of this drug, which is frequently used by the population, should be revealed in detail. This is the first detailed report to prove the negative effects of PSE. The significant decrease in the number of offspring depending on the dose increase shows us the negative effects of the drug on reproduction. We showed that the drug caused a decrease in the number of offspring depending on the

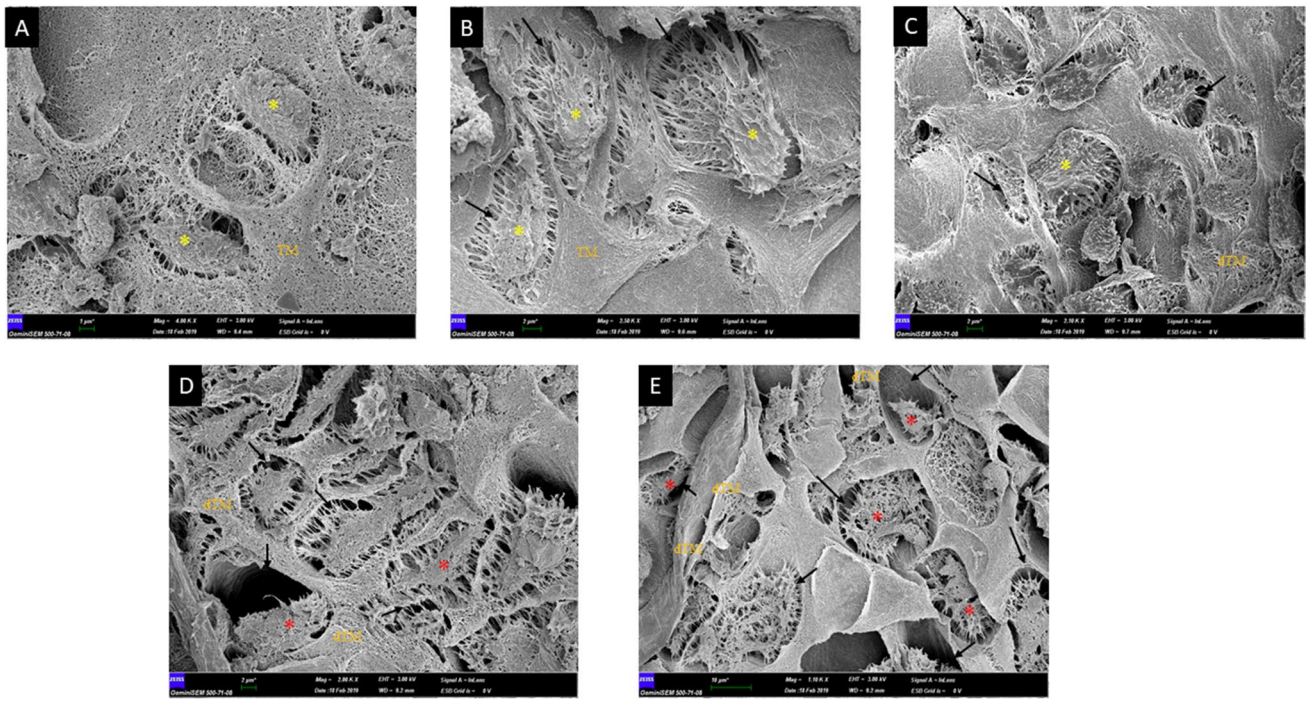

Fig. 5 Changes in chondrocytes and lacunae due to dose increase. **TM* territorial matrix, *dTM* damaged territorial matrix. **Yellow asterix indicates normal chondrocytes without severe shrank. ***Red asterix indicates severe shrank of chondrocytes. ****Black arrow indicates the gap between the chondrocyte-lacunae that occurred with the dose increase

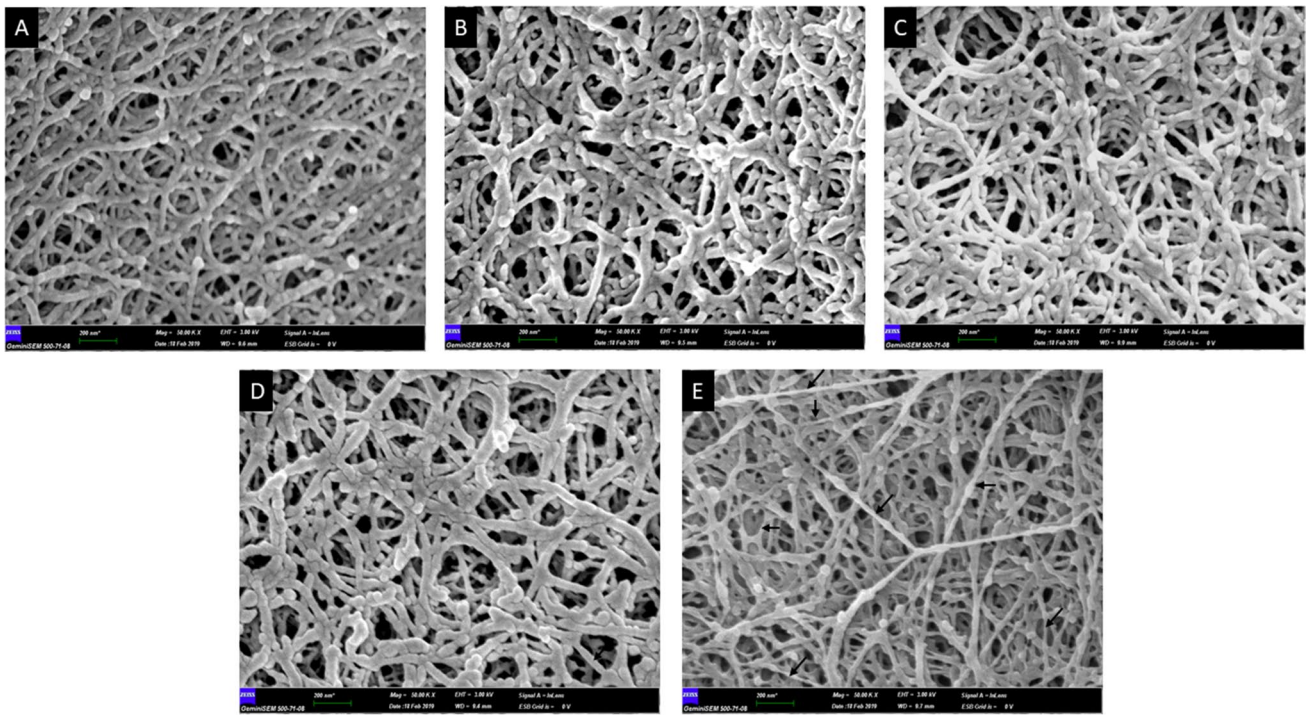

Fig. 6 Thinning of collagen fibrils due to dose increase. *The black arrow indicates severe thinning of the collagen fibril

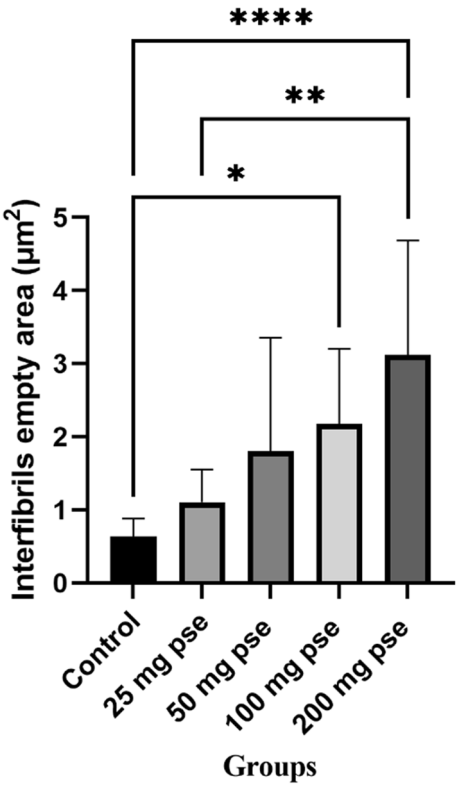

Fig. 7 Difference in the interfibrils empty space between the groups. At high doses, empty area of interfibrils increases markedly. *An one-way ANOVA test; $p < 0.05$ value was accepted as statistically significant difference

dose increase. Almost all of the fetuses; weight, length, placental weight parameters decreased with increasing dose. It is clearly seen that this growth retardation also affects bone development. While there was no adverse event in the control group pups, serious decreases were observed in morphometric parameters, especially in the pups exposed to 50 mg/kg, 100 mg/kg and 200 mg/kg PSE doses (Fig. 8). We found adverse events in the 25 mg/kg PSE group, which is the rat equivalent of the maximum safe daily dose in humans (Nair and Jacob 2016). It was observed that the embryo did not develop sufficiently in one of the fetuses exposed to high dose PSE. The placenta of this embryo was found to be above average in size (Fig. 8C). Therefore, we can state that the drug causes an increase in placental weight and abnormal growth retardation in fetal weight.

Bones are a dynamic organ that looks quite hard and robust but is constantly active to perform structural and metabolic functions. The interaction between the primary cells that make up the bone is very important in the formation of normal bone, especially by developing from cartilage cells, as in long bones. The mineralization of some bone cells with hydroxyapatite crystals using calcium and phosphate is continuous from embryonal life to death (Novack 2011). In this process, many factors regulate the development of bone from cartilage, an abnormality in these factors can affect the whole life. One of these factors is nuclear factor kappa B (NF-κB). NF-κB

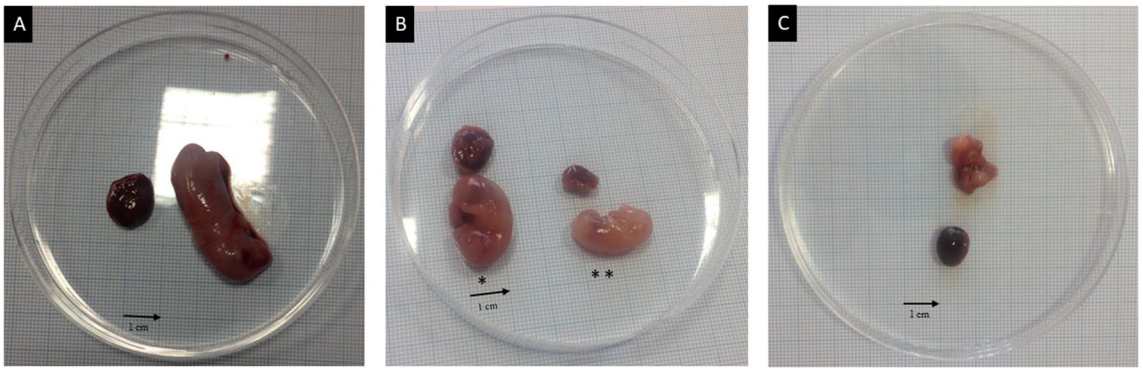

Fig. 8 Images of fetuses taken into Petri dishes. **A** Pup belonging to the control group. **B** (*) pup exposed to 50 mg/kg PSE. (**) pup exposed to 200 mg/kg PSE **C** Abnormally developed fetus exposed to 200 mg/kg PSE

is a transcription factor found in all cell types. This factor, which is inactive in the cytoplasm, takes part in bone formation. It controls the differentiation or activity of the major skeletal cell types; osteoclasts, osteoblasts, osteocytes, and chondrocytes (Novack 2011). In a study on rats, it was shown that NF-κB expressed in cartilage growth plates increased chondrocyte proliferation and differentiation, while inhibiting apoptosis, allowing the growth of long bones (De Luca 2020). In addition, another study showed that PSE inhibited NF-κB-dependent transcriptional activity (Fiebich et al. 2012). In our study, we revealed in SEM images that apoptosis intensified with the increase in PSE dose. The destruction was observed in both the territorial matrix and the interterritorial matrix due to dose increase. In addition, chondrocytes were significantly shrunk. Further studies are needed to fully reveal whether chondrocyte damage caused by exacerbation of apoptosis is caused by NF-κB.

In bone, collagen represents more than 90% of the organic matrix, and greatly influences the calcification of bone. Matrix vesicles produced by osteoblasts form crystallized hydroxy apatite (HA). With the growth of HA crystals, the membrane of the matrix vesicles ruptures and thus the surrounding areas are calcified. Calcification increases as contact with collagen fibrils increases. Therefore, collagen fibrils form calcification, albeit indirectly, like a scaffold (Hashimoto et al. 2017). In abnormal bone, collagen quality is affected (Tzaphlidou 2008). In our study, defective collagen fibril formation and architecture upon abnormalities were observed in SEM images (Fig. 6). Depending on the dose increase, deterioration and thinning of the orthogonal structure of the fibers occurred. The data of minerals obtained by EDX analysis were similar to both morphometric data of double staining, and interfibrils empty area of collagen fibrils. Depending on the dose increase, a decrease in calcium forming HA crystals and an increase in the area between collagen fibrils were observed. All these negativities in the bone were detected visibly by the double staining method (Fig. 1).

In normal healthy rat embryos, the Ca/P molar ratio is high, and it increases until the 20th day of embryonic life (Henmi et al. 2016). According to our EDX analyzes, it has been clearly shown that dose increase affects this rate negatively. In addition, the Ca/P ratio obtained from the control group was similar to the ratio obtained from human fetuses (Oyedepo and Henshaw 1997). In healthy rat embryos, a decrease in the C/Ca molar ratio is observed with increased calcification (Henmi et al. 2016). In our study, it was observed that this rate increased with the increase in dose. The increase in the C/Ca molar ratio confirmed the bone destruction due to dose. A decrease in the C/P molar ratio is expected in healthy embryos, just like the C/Ca molar ratio. However, in our study, while a decrease was observed with a small amount of dose, an increase was observed depending on the dose increase. This difference, which confirms the work of Fiebich et al., which can be explained by the lack of effect of NF-κB on the phosphorylation mechanism (Fiebich et al. 2012).

One of the limitations of our study was that histological analyzes were not performed on bone. The negative effects on the bones could be observed better with the immunohistochemical staining method. In the literature, the fetal weight of SD rats at 20.5 days was usually 3–4 g, but surprisingly, it was almost half in our study. In the literature, pregnant rats between the 6th and 15th days of pregnancy were generally treated. However, in our study, it was possible to intervene every day from the beginning to the end of the pregnancy. Therefore, we believe that this process occurring during pregnancy in animals causes low birth weights. However, we can explain with the Aliverti technique that this low birth weight does not affect bone development (Aliverti et al. 1979).

The bioavailability of pseudoephedrine varies in different organisms (Till and Benet 1979). Its bioavailability is 28%

in rats, 58% in dogs and 89% in some monkey species (Palamanda et al. 2010). In addition, the lowest dose group (25 mg/kg = rat) we used on rats in the study corresponds to the maximum safe dose that can be taken daily in humans (240 mg/day = human). The findings we obtained in our study revealed that even the lowest dose (25 mg/kg pse) had negative effects. In addition, there is no clear information in the literature about whether the drug crosses the placenta (Werler 2006; Yau et al. 2013). However, the findings of our study strengthen the perception that the drug passes through the placenta. In future studies, in order to complete some deficiencies about the drug, it should be investigated whether the drug crosses the placenta, how the differences in human bioavailability and pharmacokinetics affect the fetus.

In this study, the negative effects of PSE on rat embryos were investigated on bone. Both double staining, SEM and EDX analyzes were consistent with each other and clearly revealed the destruction of PSE on long bones. A very limited number of experimental studies have been scanned in the literature, but unfortunately the mechanism underlying this destruction has not been clearly presented. Although this study is the first to reveal the detailed effects of this drug, which is widely used by the population, we believe that the mechanism of the drug causing fetal long bones destruction should be investigated in the future. Human studies on PSE have generally been based on the survey/observation. Therefore, this study shows the characteristics of a pilot study that has not been done before in the literature. In terms of leading human studies, studies in other primates should be done first. We therefore wish our work to guide future animal and human studies.

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Author contributions YH conceived study, wrote the paper, made statistical analysis and conducted all details of study. UE conceived the study. BE made SEM experiments and analysis. UI performed gavage. DM performed cesarean section. OGO prepared chemicals. CT created graphics and helped to literature search.

Data availability statement The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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