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Morphometric investigation of the effects of Azoxymethane, Diallyl disulfide and corn oil use on humerus and femur development in rats

Investigación morfométrica de los efectos del uso de azoximetano, disulfuro de dialilo y aceite de maíz sobre el desarrollo del húmero y el fémur en ratas

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ABSTRACT

The study aimed to examine the effects of Azoxymethane (AOM), Diallyl Disulfide (DADS), and corn oil on the humerus and femur in rats. In the study, 40 male Wistar Albino rats, 12 weeks old, were used. The animals were divided into four different groups (Control, AOM, DADS and Corn oil). After the experimental period, all animals were anesthetized and sacrificed by cervical dislocation. Humerus and femur from long bones resected from all animal groups. The maximum length, proximal width, distal width, diaphysis diameter, cortex thickness and cavum medullare diameter of the bones were measured. After the morphometric measurements index 1, index 2, robusticity index, and bone weight/bone length index were calculated for the bones. No difference was observed in the morphometric measurements and indices performed on the humerus. A statistical difference in proximal width and robusticity index was detected between the groups in the femur. A difference was observed between the AOM group and the Control/corn oil groups in proximal width. A difference was detected between the AOM group and the Control group in the robustness index, and it was determined that AOM caused a decrease in density and strength in bones. It is thought that the findings obtained in this study will contribute to the evaluation of the effects of cancer research using AOM on bones in rats.

Key words: Azoxymethane; corn oil; diallyl disulfide; femur; humerus; morphometry

RESUMEN

El estudio tuvo como objetivo examinar los efectos del azoximetano (AOM), el disulfuro de dialilo (DADS) y el aceite de maíz en el húmero y el fémur de ratas. En el estudio se utilizaron 40 ratas Wistar Albinas macho, de 12 semanas de edad. Los animales se dividieron en cuatro grupos diferentes (Control, AOM, DADS y Aceite de maíz). Después del período experimental, todos los animales fueron anestesiados y sacrificados mediante dislocación cervical. Húmero y fémur huesos largos resecados de todos los grupos de animales. Se midieron la longitud máxima, el ancho proximal, el ancho distal, el diámetro de la diáfisis, el espesor de la corteza y el diámetro del cavidad medular de los huesos. Después de las mediciones morfométricas, se calcularon el índice 1, el índice 2, el índice de robustez y el índice de peso óseo/longitud ósea para los huesos. No se observaron diferencias en las mediciones e índices morfométricos realizados en el húmero. Se detectó una diferencia estadística en el ancho proximal y el índice de robustez entre los grupos en el fémur. Se observó una diferencia entre el grupo AOM y los grupos Control/aceite de maíz en el ancho proximal. Se detectó diferencia entre el grupo AOM y el grupo Control en el índice de robustez, y se determinó que la AOM provocó una disminución en la densidad y fuerza en los huesos. Se cree que los hallazgos obtenidos en este estudio contribuirán a la evaluación de los efectos de la investigación del cáncer utilizando AOM en huesos de ratas.

Palabras clave: Azoximetano; aceite de maíz; disulfuro de dialilo; fémur; húmero; morfometria

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INTRODUCTION

The organic substances that make up the bone form the elasticity of the bone, and the inorganic substances form the hardness and durability of the bone [1, 2]. As progressive aging, the amount of organic substance in the bones decreases, and the amount of inorganic substance increases. For this reason, bone fractures are more common in older animals [1]. This development and alteration in bones is frequently investigated through experimental studies in rats (*Rattus norvegicus*) [3, 4, 5, 6, 7].

Azoxymethane (AOM) is frequently used to create cancer models in experimental animals. It has been reported that cancer models created with AOM are similar to cancer observed in humans [8]. It is stated that AOM induction changes the metabolic balance of the intestinal epithelial layer and produces hydrogen peroxidase [9].

It is reported that garlic, which is known to have cancer prevention properties, reduces stomach, breast, colon, esophagus and ovarian cancers when used regularly [10, 11]. It has been stated that garlic and garlic extracts have anti-cancer properties in animal models [12]. Diallyl disulfide (DADS), which is light yellow in color and liquid with a pungent garlic odor, is one of the oil-soluble organosulfur compounds of garlic. DADS is non-polar and insoluble in water. Due to this feature, it can be dissolved in non-polar solvents [13, 14]. Therefore, DADS is applied by dissolving it in corn oil. Garlic and its components, in addition to being antioxidants, also have antibiotic, antiviral, antifungal, antihistamine, and anti-parasitic properties [15, 16, 17]. Corn oil is obtained from Zea mays corn grains from the Graminae family. Corn oil contains high amounts of unsaturated, low saturated fatty acids and high amounts of vitamin E [18, 19].

Morphometry is one of the most important methods used to measure length, width and thickness of biological structures such as bones [20, 21]. Differences are revealed with the measurements and statistical analyzes obtained with this method [20]. Morphometric measurements in bones are made directly with a digital caliper. In recent years, morphometric measurements have also been made on 3D models created using devices such as three-dimensional laser scanners and computerized tomography [22, 23].

Morphometric measurements made on the front and hind leg bones of rats have been used in sexual dimorphism as in other animals [24, 25]. It is reported that audiogenic stress negatively affects long bone development in rats and significantly reduces bone length [26]. The effect of Methenolone Enanthate (ME), one of the Anabolic-androgenic Steroids (AAS), on femur development was examined in adolescent rats. In the study, it was observed that ME application negatively affected femur length and corpus femoris thickness in male rats. [27]. It has been reported that humerus and femur lengths were shortened in rats supplemented with boldenone, trenbolone and testosterone [3, 5, 7].

In addition to the length measurement obtained from the bones, it is stated that the robusticity increases at the same rate as the robusticity index obtained from the bones decreases [24, 25, 28]. The effects of lindane and linuron on bone morphemetry, calcium mechanism and kidneys were examined in rats. It has been stated that the use of high doses of linuron causes a decrease in the density and strength of the femur [29]. Bone mineral content (BMC) and bone mineral density (BMD) were examined in rats by dual-energy X-ray absorptiometry (DXA) (Norland. Fort Atkinson. Wisc. USA). In the study comparing DXA and histomorphometry with two morphometric indices (bone robusticity and bone weight/bone length), it was determined that morphometric indices were closely related to BMC [30]. It has been observed that smoke inhalation has a negative effect on the skeletal system during the development period, and the strength of bones decreases in rats exposed to smoke inhalation [31]. Similarly, it has been stated that estrogen deficiency from ovariectomy causes bony tissue changes in the femur length. Accordingly, it has been determined that the density and strength of the femur decreases [32, 33, 34, 35].

The objective of the study is to morphometrically examine the effects of AOM, DADS and Corn oil on the humerus and femur, which are long bones, in rats. It is known that there is limited information on the effects of AOM (It is frequently used to create cancer models in experimental studies), DADS (It is one of the oil-soluble organosulfur compounds of garlic), and Corn oil on long bones in the rat.

MATERIALS AND METHODS

In the study, 40 male Wistar Albino rats, 12 weeks old, were used. During the study, animals were accommodated in environmental conditions of 40% humidity and 20°C, 12 hours at night and 12 hours during the day. The study was approved by the Ethics Committee of Selçuk University Faculty of Veterinary Medicine (Approval number: 2023/110).

Experimental design

The animals were divided into 4 different groups. In the first group, the Control group (n=10) the feed and water needs of the animals were supplied ad libitum during the experiment (18 week). AOM group (n=10) was fed a standard rat diet and 15 mg·kg^{-1} body weight AOM (Azoxtmehane Sigma-Aldrich) was injected subcutaneously (SC) 2 times for two weeks [36]. DADS group (n=10) was fed a standard rat diet and 50 mg·kg^{-1} body weight DADS was administered for the last 3 weeks [37]. Since DADS is non-polar and insoluble in water, it was applied by dissolving it in corn oil. The corn oil group (n=10) was fed a standard rat diet and 1 mg·kg^{-1} body weight corn oil was administered for the last 3 weeks.

Morphometric measurement

All animals were fed ad libitum during the experimental process (18 weeks). After the experimental period, all animals were anaesthetised with Ketamine (95 mg·kg^{-1}, SC) + Xylazine (5 mg·kg^{-1}, SC) and sacrificed by cervical dislocation. Left humerus and femur resected from all groups of animals. Since the effect of different substances used on bone development was investigated in the study, the left/right factor in bones was not taken into account. The soft tissue on the humerus and femur of the rats was removed (FIG. 1).

After removing the soft tissue from the bones, measurements were made using a 0.01 mm accuracy digital calliper (FIG. 2). Morphometric measurement points performed on the femur and humerus are specified in TABLE I. Morphometric measurements were carried out from the measurement points applied in previous studies [4, 5, 7, 38, 39, 40]. The points of the morphometric measurements of the humerus specified in TABLE I are shown in FIGS. 3.

In measuring the diaphysis diameter of the humerus, the lower border level of the tuberosity deltoidea was considered. In measuring the cortex thickness of the humerus, the average of four different measurements of compact bone tissue was taken. In measuring the cavum medullare diameter of the humerus, the mediolateral diameter was considered as stated in previous studies.

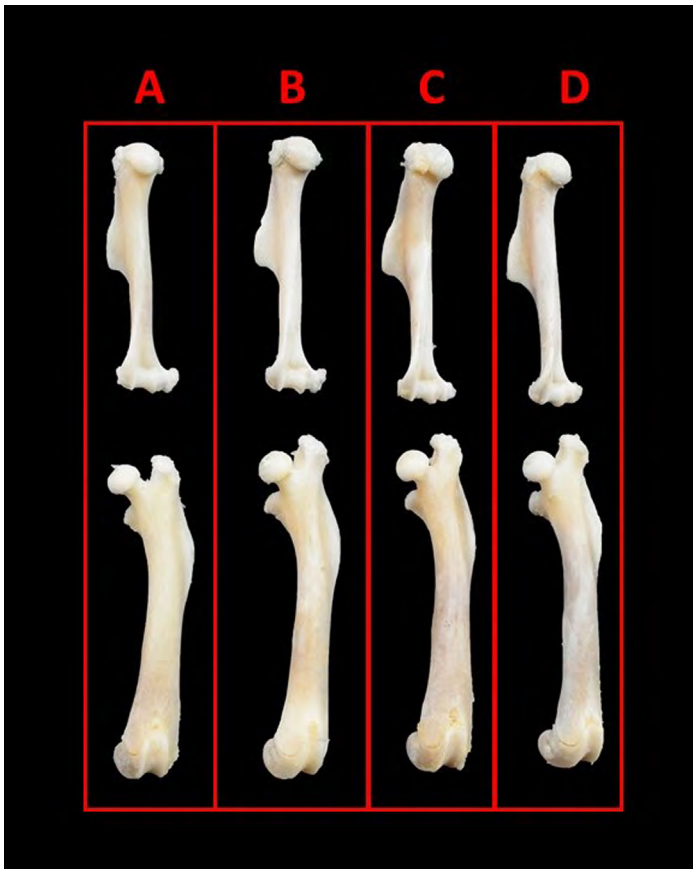

FIGURE 1. Rat humerus and femur bones. A: Control group, B: AOM group, C: DADS group, D: Corn oil group

TABLE I
Morphometric measurements of humerus and femur

Parameters	
Weight of bone	Diaphysis diameter
Maximum length	Cortex thickness
Proximal width	Cavum medullare diameter
Distal width	

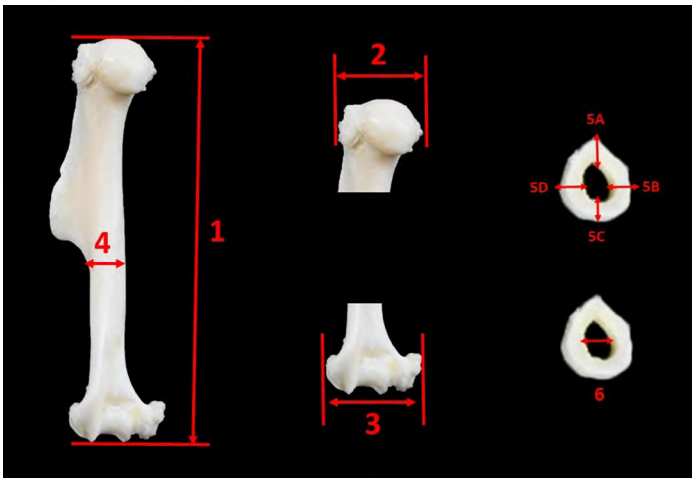

FIGURE 3. Maximum length of the humerus (1), Proximal width of the humerus (2), Distal width of the humerus (3), Diaphysis diameter of the humerus (lower border level of the tuberositas deltoidea) (4), Cortex thickness of the humerus {(5A+5B+5C+5D)/4} (5), Cavum medullare diameter of humerus (6)

The points of the morphometric measurements of the femur specified in TABLE I are shown in FIG. 4.

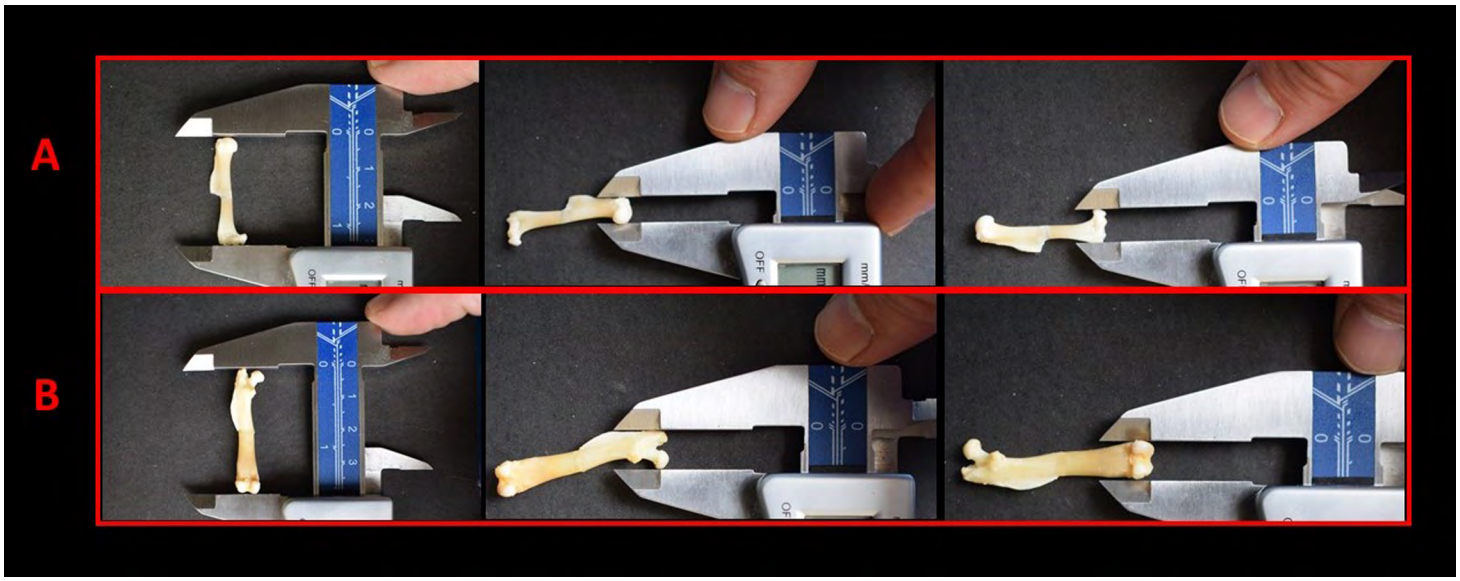

FIGURE 2. Morphometric measurement with digital calliper. A: Humerus, B: Femur

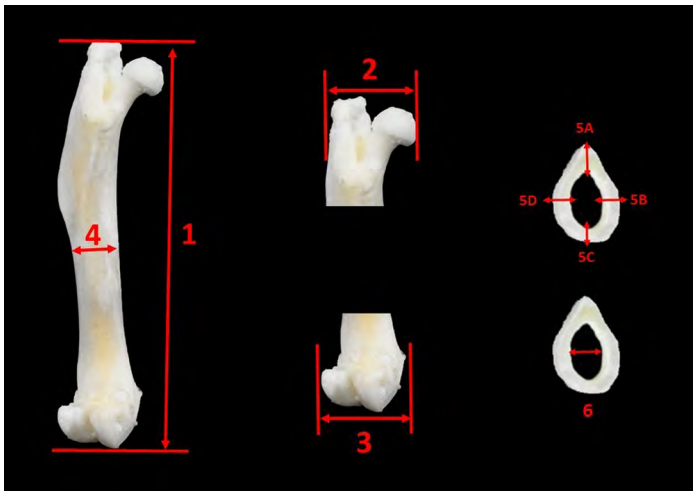

FIGURE 4. Maximum length of the femur (1), Proximal width of the femur (2), Distal width of the femur (3), Diaphysis diameter of the femur (lower border level of the trochanter tertius) (4), Cortex thickness of the femur {(5A+5B+5C+5D)/4} (5), Cavum medullare diameter of femur (6)

In measuring the diaphysis diameter of the femur, the lower border level of the trochanter tertius was considered. The points mentioned in the humerus were taken into account when measuring the cortex thickness and cavum medullare diameter of the femur.

The following equations of index 1, index 2, robusticity index, and bone weight/bone length index were calculated with the morphometric measurements obtained from the bones [24, 28, 30, 40, 41, 42]. The lower the index of robusticity, the denser and stronger the bone. The higher the index of robusticity, the less dense or more porous the bone [24, 42]. The bone weight/bone length index is a simpler index of bone density used by Seedeor [41].

$$Index\ 1 = \frac{Diaphysis\ diameter - Cavum\ medullare\ diameter}{Diaphysis\ diameter} \times 100$$

$$Index\ 2 = \frac{Diaphysis\ diameter}{Maximum\ length} \times 100$$

$$Robusticity\ index = \frac{Maximun\ length}{\sqrt[3]{Weigth\ of\ bone}}$$

$$Bone\ weight\ /\ bone\ length\ index = \frac{Weight\ of\ bone}{Maximun\ length}$$

The data were evaluated in the statistical package program IBM SPSS Statistics Standard Concurrent User V 26 (IBM Corp., Armonk, New York, USA). Descriptive statistics are given as mean ± standard deviation values. The homogeneity of the variances was tested using the Levene test. Normal distribution of the data of numerical variables was evaluated with the Shapiro Wilk normality test. One-way analysis of variance was used if the data were normally distributed in the variables, and the Kruskal Wallis H Test was used if the data were not normally distributed (It was done only on the proximal width of the femur parameter). Tukey test was used as multiple comparison tests when the result of analysis of variance was significant. $p<0.05$ was considered statistically significant. "Nomina Anatomica Veterinaria" was used for anatomical terms [43].

RESULTS AND DISCUSSION

Humerus demographic results of all groups obtained from morphometric measurements (weight of bone, maximum length, proximal width, distal width, diaphysis diameter, cortex thickness, cavum medullare diameter, index 1, index 2, robusticity index and bone weight/bone length index) are summarized in TABLE II.

TABLE II
Descriptive statistics of parameters measured from the humerus (Mean ± SD)

Parameters	Statistics
Weight of bone	0.45 ± 0.03
Maximum length	31.41 ± 0.62
Proximal width	6.20 ± 0.19
Distal width	7.60 ± 0.23
Diaphysis diameter	2.95 ± 0.11
Cortex thickness	0.93 ± 0.07
Cavum medullare diameter	1.14 ± 0.18
Index 1	61.27 ± 5.97
Index 2	9.38 ± 0.34
Robusticity index	4.09 ± 0.07
Bone weight/bone length index	14.47 ± 0.91

It was observed that the humerus lengths remained short in rats administered different doses of boldenone, trenbolone, and testosterone supplementation. Similarly, the use of testosterone supplementation in young swim-trained rats causes shortening in extremity bones [3, 5, 7]. In the study using AOM, DADS, and corn oil, no difference in the length of the humerus was observed. Similarly, It was observed that the use of *Tarantula cubensis* alcoholic extract (TCAE) did not affect the length of the humerus.

Intergroup comparison of morphometric measurement results and indices obtained from the humerus was presented in TABLE III. No difference was observed between the Control, AOM, DADS, and Corn oil groups in the measurements applied to the humerus.

In the indices obtained from the humerus, the highest values for index 1 and index 2 were observed in the corn oil group. The highest value in the bone weight/bone length index was observed in the AOM group. In the robusticity index, where the index value and robusticity are inversely proportional, the smallest value was calculated in the AOM and DADS groups.

Femur demographic results of all groups obtained from morphometric measurements are summarized in TABLE IV.

It was observed that the femur lengths remained short in rats administered different doses of boldenone [3]. In another study examining the effect of trenbolone supplementation on the extremity bones of running rats, it was observed that the femur lengths were short in rats [5]. Similarly, it has been reported that the use of testosterone supplementation in young swim-trained rats causes shortening in extremity bones [7]. In the current study, while no difference was observed in the lengths of the femur, a difference

TABLE III
Comparison of humerus parameters and indices between groups

Parameters	Groups				Test Statistics	*P*
	Control	AOM	DADS	Corn oil		
Weight of bone (g)	0.46±0.04	0.46±0.04	0.45±0.02	0.45±0.04	0.390	0.761[x]
Maximum length (mm)	31.67±0.66	31.37±0.70	31.20±0.39	31.42±0.69	1.009	0.400[x]
Proximal width (mm)	6.23±0.27	6.15±0.14	6.19±0.11	6.24±0.22	0.395	0.757[x]
Distal width (mm)	7.49±0.20	7.62±0.24	7.73±0.29	7.57±0.16	1.947	0.139[x]
Diaphysis diameter (mm)	2.94±0.08	2.94±0.17	2.93±0.08	2.97±0.07	0.226	0.878[x]
Cortex thickness (mm)	0.92±0.05	0.94±0.08	0.91±0.06	0.95±0.07	0.563	0.643[x]
Cavum medullare diameter (mm)	1.22±0.11	1.15±0.18	1.09±0.23	1.10±0.18	1.005	0.402[x]
Index 1	58.68±3.28	60.78±5.86	62.72±7.44	62.88±6.40	1.100	0.362[x]
Index 2	9.30±0.28	9.38±0.52	9.40±0.27	9.46±0.25	0.351	0.788[x]
Robusticity index	4.10±0.07	4.07±0.08	4.07±0.06	4.11±0.08	0.955	0.424[x]
Weight / length index	14.55±0.98	14.66±1.06	14.43±0.68	14.23±0.96	0.393	0.759[x]

Numerical variables are given as mean ± standard deviation. [x]: One–way ANOVA, Row–based lettering was done. There is no difference between the same letters. In the index equations, the weight is taken as milligrams

TABLE IV
Descriptive statistics of parameters measured from the femur (Mean±SD)

Parameters	Statistics
Weight of bone	1.02±0.07
Maximum length	41.66±0.91
Proximal width	9.20±0.32
Distal width	7.38±0.18
Diaphysis diameter	5.14±0.18
Cortex thickness	1.00±0.08
Cavum medullare diameter	3.07±0.18
Index 1	40.26±3.38
Index 2	12.35±0.35
Robusticity index	4.14±0.06
Bone weight/bone length index	24.49±1.33

was detected in the proximal width of the femur. It was observed that the proximal width of the femur increased in rats given AOM.

Audiogenic stress is known to affect osteogenesis in rats, and bone length negatively decreases significantly. It is reported that ME, one of the Anabolic–Androgenic Steroids, negatively affects femur development and negatively affects femur length. Similarly, it was observed that the humerus and femur lengths decreased significantly in rats given Methenolone Enanthate Supplement (MES) along with exercise [6, 26, 27]. In the study using AOM, DADS, and corn oil, no difference in the length of the femur was observed. However, it has been observed that AOM negatively affects bone robusticity.

In another study, the effect of TCAE, which is used as a homeopathic medicine in etherine medicine, on bone development in rats was examined [4]. In the study where it was used in young rats, no difference was observed in the measurements made on the femur, as in the current study.

Intergroup comparison of morphometric measurement results and indices obtained from the femur was presented in TABLE V.

There is a statistically significant difference between the groups in the variables proximal width and robusticity index ($P<0.05$). There is a difference between the AOM group and the Control/corn oil groups in the proximal width parameter. The mean of the AOM group is higher than the Control and corn oil groups. There is a difference between AOM and Control groups in the robusticity index. The mean of the AOM group is higher than the mean of the Control group.

In the indices obtained from the femur, the highest values for index 1 was observed in the AOM group. The highest values for index 2 was observed in the DADS group. The highest value in the bone weight/bone length index was observed in the Control group. In the robusticity index, where the index value and robusticity are inversely proportional. There is a difference between AOM and Control groups. The highest robusticity index was observed in the AOM group. The use of AOM negatively affects bone density.

Robusticity index, which is an important value in determining bone density and porosity, was calculated in healthy female and male Wistar albino, Wild rats and Sprague–Dawley rats [25]. The robusticity index using different strains was calculated slightly higher than the values obtained in the current study. It is thought that this difference is due to the age of wild rats (ages unknown) and all domesticated rats (6 months) used in the relevant study.

Indices are calculated using morphometric measurements made on bones. The robusticity index is one of the most frequently used indices in research on rat bones. The lower the index, the denser or more robust the bone [24, 25, 28]. In a study investigating the effects of lindane and linuron on rats, it was determined that both compounds did not affect the weight and length of the femur. However, it has been stated that the use of high doses of linuron causes a decrease in density and strength in the femur [29]. Similarly, it has been determined that the use of AOM causes a decrease in density and strength in the femur. Additionally, it has been reported that smoke inhalation and estrogen deficiency from ovariectomy in rats negatively affect bone development [32, 33, 34, 35].

TABLE V
Comparison of femur parameters and indices between groups

Parameters	Groups				Test Statistics	*P*
	Control	AOM	DADS	Corn oil		
Weight of bone (g)	1.04±0.10	1.00±0.08	1.03±0.04	1.02±0.06	0.472	0.703[x]
Maximum length (mm)	41.64±1.44	41.84±0.71	41.60±0.60	41.56±0.79	0.169	0.917[x]
Proximal width (mm)	9.04±0.39[a]	9.40±0.06[b]	9.23±0.26[ab]	9.15±0.37[a]	10.685	**0.014[€]**
Distal width (mm)	7.32±0.27	7.41±0.14	7.38±0.18	7.41±0.12	0.522	0.670[x]
Diaphysis diameter (mm)	5.05±0.22	5.18±0.17	5.18±0.13	5.17±0.16	1.220	0.316[x]
Cortex thickness (mm)	0.99±0.06	1.00±0.06	0.98±0.10	1.04±0.09	0.937	0.433[x]
Cavum medullare diameter (mm)	3.06±0.21	3.04±0.15	3.05±0.24	3.14±0.12	0.564	0.642[x]
Index 1	39.43±3.51	41.20±3.16	41.05±4.78	39.35±1.04	1.692	0.639[€]
Index 2	12.14±0.41	12.37±0.37	12.45±0.34	12.44±0.21	1.805	0.164[x]
Robusticity index	4.11±0.04[a]	4.19±0.07[b]	4.13±0.06[ab]	4.13±0.06[ab]	3.175	**0.036[x]**
Weight / length index	24.91±1.46	23.91±1.63	24.67±0.93	24.47±1.17	1.040	0.386[x]

Numerical variables are given as mean ± standard deviation. [x]: One-way ANOVA, [€]: Kruskal-Wallis test, Row-based lettering was done. There is no difference between the same letters. In the index equations, the weight is taken as milligrams

CONCLUSION

In this study, the effects of AOM, DADS and corn oil on bone development were investigated, and morphometric measurements were performed on the long bones humerus and femur. A difference in the proximal width of the femur was observed in morphometric measurements. According to the indices obtained from the measurement results, it was observed that the use of AOM reduced the density and strength in the femur. No difference was observed in DADS and corn oil. It is thought that the findings obtained in this study will contribute to considering the effects of cancer studies on bones in rats.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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