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Diazinon exposure reduces bone mineral density in adult and immature rats: A histomorphometric and radiographic study

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Abstract

Diazinon has been widely used as a domestic and agricultural pesticide. This study examined the effects of diazinon on bone mineral density (BMD) of mature and immature rats. For this purpose, 24 adult Wistar rats (male; 8 weeks old) were initially divided into four groups ($n = 6$). Corn oil was used as the control while diazinon at 15, 30, and 45 mg/kg in corn oil was given to mature rats via gavage per day. Since these dosages were lethal for the immature rats, 12 immature Wistar rats (male; 4 weeks old) ($n = 6$) were gavaged with corn oil as control and 5 mg/kg of diazinon in corn oil. The animals were sacrificed on day 28 with their left femur bones removed for histomorphometric studies. BMD was measured in the right femur, using standardized radiographs in the femoral head, femoral neck, greater trochanter, and shaft. The Image J Program was used for measuring the bone lamellae and epiphyseal growth plates. The results of this study for the first time revealed that diazinon reduced BMD in both adults and immature rats. Diazinon exposure was associated with diminished trabecular and cortical bone density. Correspondingly, our results indicated that in immature rats, DZN led to the reduction in the epiphyseal growth plate width, both in the proliferation and hypertrophic zones. These results suggested that diazinon might be associated with impaired bone longitudinal growth as well as bone metabolism in adults.

Keywords

Animal, epiphyseal growth plate, bone, toxin, osteoporosis

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Introduction

Pesticide residues are one of the main concerns for environmental pollution. Organophosphorus (OPs) insecticides are widely used and shown to persist in water, soil, fruits, vegetables, grains, and other food products (Galloway, 2006). Diazinon (*O*, *O*-diethyl-*O*-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate) (DZN) is an organophosphate insecticide; although since 2004, the United States Environmental Protection Agency banned the domestic usage of diazinon, it is still widely used in agriculture and even for domestic purposes in developing countries (Montuori et al., 2015). Some features of diazinon, including moderate mobility and solubility in water, and its stability in the soil have made it a hazardous pollutant for surface water and groundwater (Farhadi et al., 2021). Various environmental and pathological effects of diazinon have been demonstrated (Aggarwal et al.,

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2013). DZN can be absorbed into the body primarily through the skin or eye contact, as well as respiration and ingestion. It has been shown that DZN may increase oxidative stress, along with histopathological and biochemical changes in the body in a dose-dependent manner (Azmi et al., 2006; Kalender et al., 2006).

When diazinon enters the body, a cleavage of the P-O-pyrimidine group followed by oxidation and dealkylation of the alkyl substituents on the pyrimidine ring have been reported (Earl et al., 1971). Diazinon is then oxygenated to an intermediate compound, phosphooxythiran, which is then desulfurized to form diazoxon (Pourtaji et al., 2016), an organophosphate compound that is much more toxic than diazinon in inhibiting acetylcholinesterase (AChE) (Kalender et al., 2005).

Studies have indicated that diazinon in nonlethal doses could cause a range of cellular and physiological damages in different animal species. The effects of this toxicant have been shown on the nervous system, liver cells, kidney cells, germ cells, and gonads. The severity of damages depends on different factors such as dosage and exposure time as well as its stability in the body (Aggarwal et al., 2013; Harchegani et al., 2018).

The growth plate is a very organized cartilage between the epiphysis and diaphysis of long bones, which is divided into two main different horizontal phases of proliferation and hypertrophic zones (Van der Eerden et al., 2003). Bone longitudinal growth is the result of chondrocyte proliferation and subsequent differentiation in the epiphyseal growth plates of the long bones. It is regulated by a multitude of genetic and hormonal factors, growth factors, environment, and nutrition (Upledger, 2005). Under normal conditions, along with sexual maturity, growth plates fuse (chondrocyte matrix changes to osteoid); so the longitudinal growth of bone ceases (Van der Eerden et al., 2003).

Studies on the effects of diazinon on bone tissue and skeletal systems are very limited. It has been shown that diazinon could have teratogenic effects on the growth of bone and cartilage in the chick and quail embryos. DZN also reduced growth and development of foot, wing, and caused twisted claws. Reduced calcification was also observed in the leg bones (Meneely and Wyttenbach, 1989; Misawa et al., 1982). In another study, accidental exposure of a family to this poison caused adverse effects on the growth of the musculoskeletal system in addition to neurotoxicity and endocrine toxicity. In the children of this family, delays in bone growth, reduced calcification, development of cysts in the bones, pathological fractures, and bone graft failure were observed (Dahlgren et al., 2004). Previously, we have published an initial report that DZN may cause osteoporosis (Lari et al., 2011).

In this study, for the first time, we have examined the effects of DZN on bone density of different ages of rats and reported that DZN exposure is associated with reduced bone mineral density (BMD) in both adult and immature rats. Also in immature rats, DZN led to a reduction in the epiphyseal growth plate width.

Materials and methods

Animals

This study was performed on male Wistar rats, adult (8 weeks old, 250–300 g) and immature (4 weeks old; 100 g). The rats were maintained in a room at $23 \pm 1^{\circ}C$, with a fixed 12 h artificial light cycle, and they were permitted to eat and drink ad libitum. The whole process of the study was carried out in compliance with the guidelines for the care and use of Laboratory Animals published by the National Institutes of Health (NIH Publications No.8023, revised 1978). The adult rats were divided into four groups, each group consisting of six animals. Group 1 was the control group in which corn oil was administered (2 mL/kg) orally; groups 2–4 were given three different doses of DZN (95%, Tosco Chemical Co. Shanghai, China) (15, 30, and 45 mg/kg in corn oil orally once a day), for 28 days. When the immature rats were exposed to the above dosages of diazinon, even at 15 mg/kg, none of them survived. Thus, we reduced the doses to 5 mg/kg, which was the nonlethal dose for these animals. The substances were administered in the morning (between 09:00 and 10:00 h). All the animals were weighed prior to the launching of the experiments and on day 28 of the treatments. At the end of day 28, animals were euthanized and the left and right femurs were removed.

Radiography analysis

BMD was measured in the right femur after removing the soft tissues around the bone. For radiography densitometry, lateral radiographs of the femur were obtained using an X-ray apparatus and Kodak® high-resolution mammography films. An aluminum

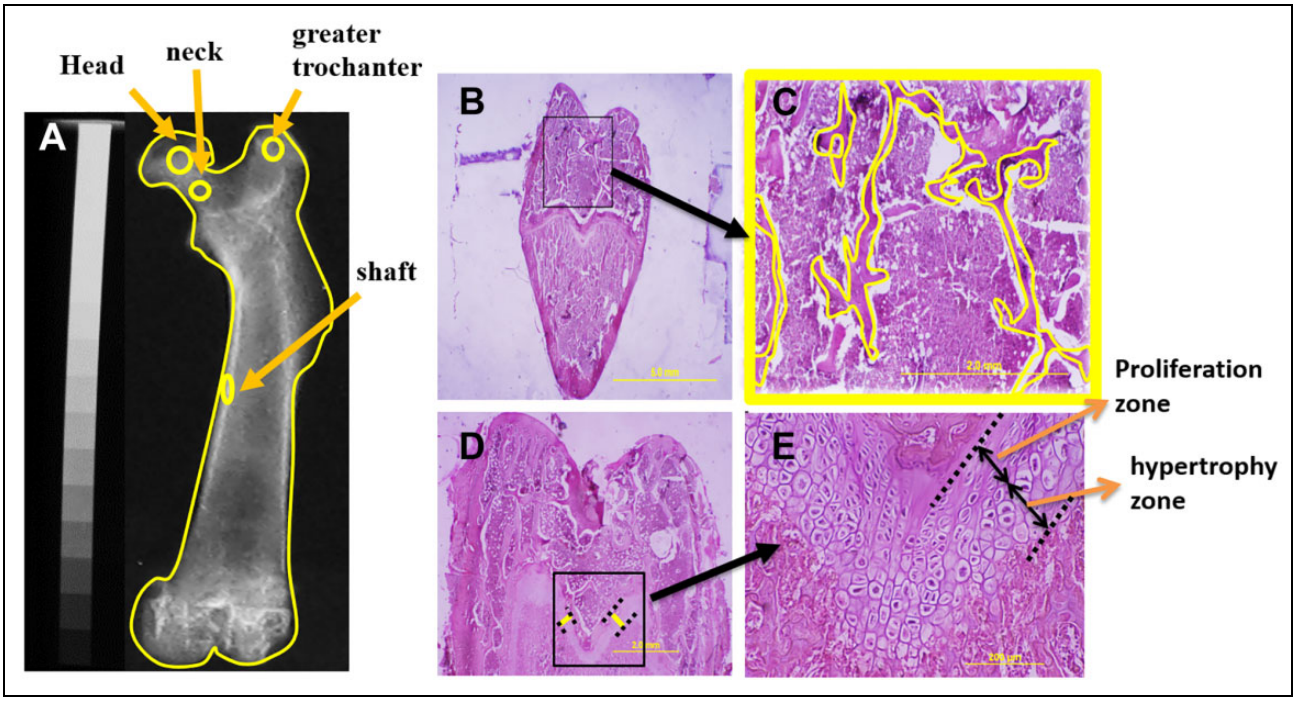

Figure 1. Radiography and histomorphometric analysis of bone and growth plate: (A) radiographic bone aluminum equivalence (0.25 mm Al) and selected sites for measuring bone density, (B, C) measurement of the bone lamellae in H&E-stained histological sections, (D, E) measurement of the width of the epiphyseal growth plate, proliferation, and hypertrophy zones in H&E-stained histological sections. H&E: hematoxylin and eosin.

step-wedge consisting of 15 steps with increasing 0.25 mm thickness of the steps was placed in a fixed position on a radiography cassette at the same level for each exposure and a single pulse snapshot was digitized by computer. The aluminum wedge is reported to have absorption and scattering properties that are similar to bone (Kinds et al., 2011; Takaishi et al., 2010) and used for bone densitometry (Boivin and Meunier, 2002; Farlay et al., 2019). The final image was assessed and calibrated using Image-J software (Kinds et al., 2011; Takaishi et al., 2010). Mean pixel intensities were measured for each step by placing a square region of interest repeatedly over the image of the step-wedge. To avoid slight changes caused by nonspecific pixels, the square was placed on a blank screen (no bone or step wedge) and regarded as 0 mm aluminum. A nonlinear calibration curve (third-degree polynomial) was produced. All subsequent measurements made on each calibrated image were based on millimeters of aluminum equivalent (mm Al. equi). Using polygon selection tools of the software, a region of femoral bone was selected, with the mean radiographic density measured for each bone and expressed as mm Al. equi (Figure 1(A)).

Histomorphometric analysis

The left femur of animals was fixed in 10% formalin for 24 h, decalcified in 7% nitric acid with a daily refill of solution for 5 days. The sodium sulfate solution was used to neutralize nitric acid. The bones were washed in running water to remove the trace of sodium sulfate, with the sections stained with hematoxylin and eosin (H&E).

Histomorphometric analysis was performed on sections that were cut from the proximal end of the epiphyseal left femurs and stained with H&E. The lamellae surface, the width of the epiphyseal growth plate, proliferation, and hypertrophic zones were measured separately as described previously (Parfitt et al., 1987). Briefly, six different sections of each group were used to measure the lamellae and width of the epiphyseal growth plate using Image J software (Wayne Rasband, NIH, USA) (Figure 1(B) and (C)). In the epiphyseal growth plate, three areas of each section were measured on photographs prepared from microscopic sections; the average size was considered as epiphyseal growth plate width. Proliferation and hypertrophic zone widths were measured separately as described earlier

(Figure 1(D) and (E)) (Parfitt et al., 1987; Rajpar et al., 2009; Yoon et al., 2019).

Statistical analysis

The data were presented as mean ± SD. Results were analyzed using unpaired two-tailed *t*-test and one-way analysis of variance Tukey's test with GraphPad Prism version 8.0 (GraphPadSoftware Inc., San Diego, CA, USA). A p value of <0.05 was considered statistically significant.

Results

Evaluation of body weights

Although all the animals in the study groups gained weight during the experimental period, body weight did not show any significant difference between DZN-treated groups and the control group (Table 1).

Table 1. The effect of DZN on body weight.[a]

Mature rats				
D 0	Con	15	30	45
277.5 ± 25	299.1 ± 28.4	309 ± 21.6	326.1 ± 8.58	295.8 ± 15.2
Immature rats				
D 0	Con	5		
93.9 ± 13.4	203.7 ± 11.7	198.45 ± 10.5		

DZN: diazinon.

[a] There was no significant difference between DZN-treated groups and the control group.

Radiology assessment of bone density

Radiology assessment of the total femur density in adult rats showed a significantly lower density for DZN groups of 15 mg/kg ($p < 0.01$), 30 mg/kg ($p < 0.001$), and 45 mg/kg ($p < 0.05$) when compared with the control group (Figure 2). The reduction of total femur density caused by DZN was observed similarly in immature rats ($p < 0.05$) (Figure 2).

The analysis of the radiographic femoral bone density of head, greater trochanter (GT), neck, and femoral shaft (FS) in adult rats indicated that DZN did not have any significant effects on the head and neck. However, at 30 and 45 mg/kg dosage, DZN significantly reduced FS ($p < 0.05$) and GT ($p < 0.01$). Also, DZN at 15 mg/kg lowered the bone density of FS ($p < 0.05$) (Figure 3).

In immature rats, the results were more dramatic, that is, DZN significantly reduced head, GT, and neck ($p < 0.001$) as well as FS ($p < 0.05$) (Figure 3).

Histological assessments

Measurement of the percentage of lamellae surface in mature rats indicated that the application of 30 mg/kg ($p < 0.05$) and 45 mg/kg ($p < 0.0001$) of DZN significantly reduced the lamellae percentage in the epiphyseal bone. This reduction was seen more intensely in immature rats ($p < 0.01$) (Figure 4).

Since DZN, even at a lower dosage, had a great impact on the bone of immature rats, the histological effect of DZN on the growth plate of immature rats was also evaluated. The average widths of the growth plate, proliferating zone, and hypertrophic zone were

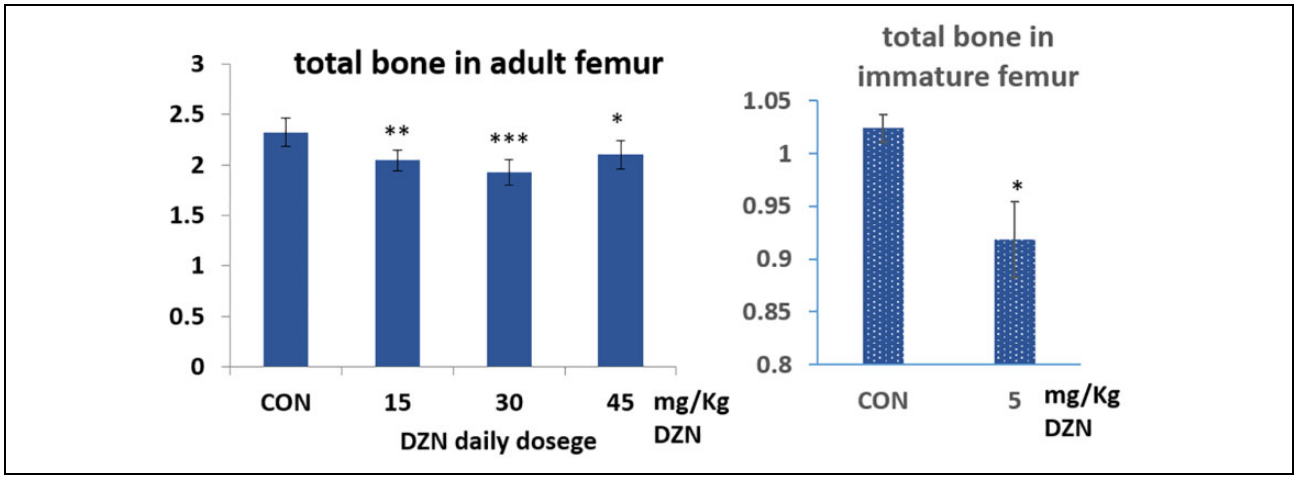

Figure 2. Radiology assessment of the total femur bone density in adult and immature rats treated with diazinon ($n = 6$). Values are given as mean ± SD. *$p < 0.05$, **$p < 0.01$ versus control group. DZN: diazinon; CON: control.

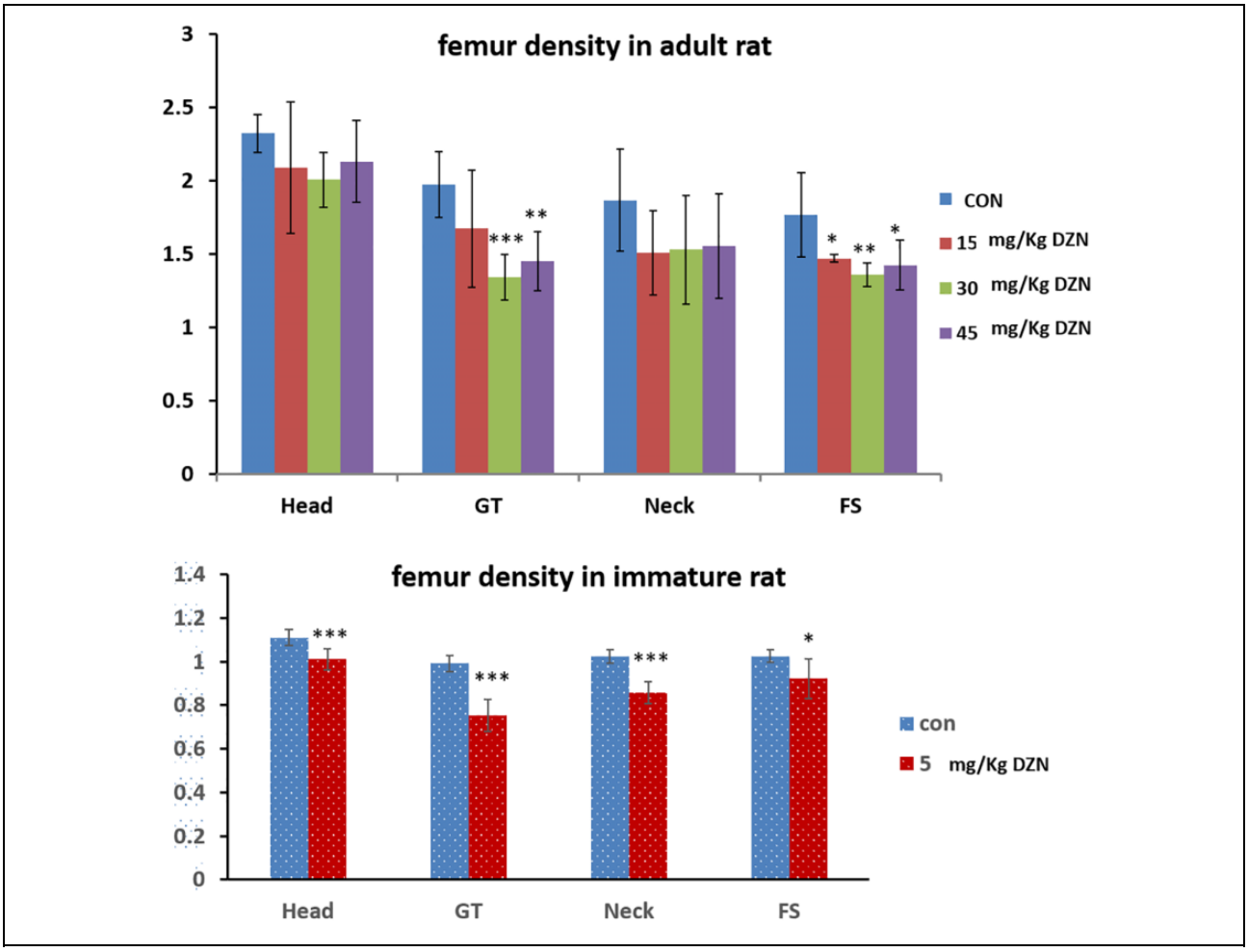

Figure 3. Radiology assessment of the bone density of head, GT, neck, and FS in the femur of adult and immature rats treated with diazinon ($n = 6$). Values are given as mean $\pm$ SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control group. DZN: diazinon; CON: control; GT: greater trochanter; FS: femoral shaft.

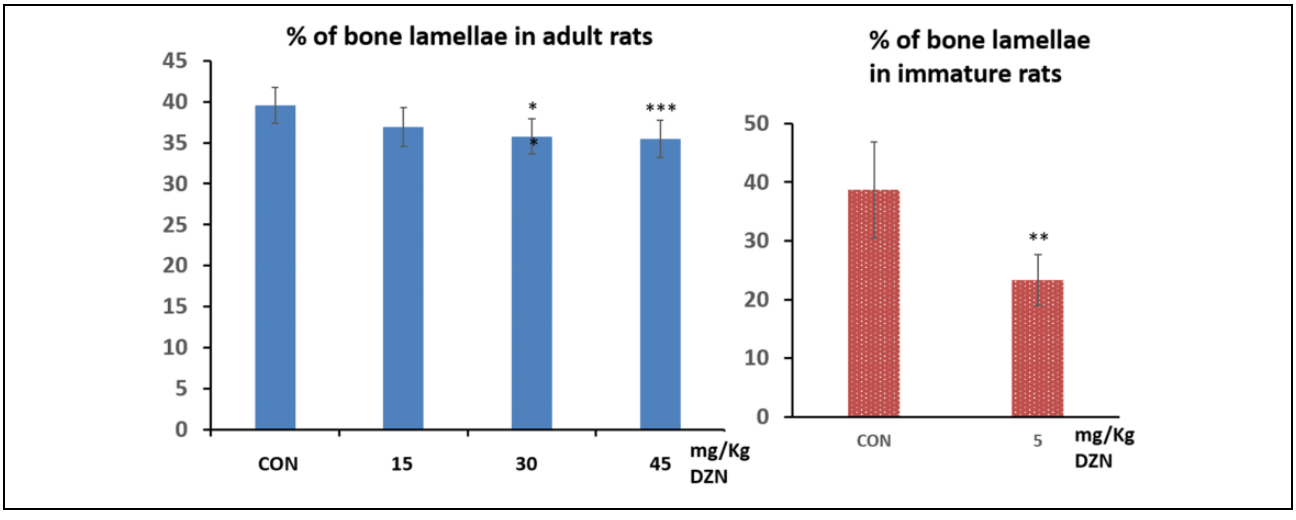

Figure 4. The percentage of lamellae surface in adult and immature rats treated with diazinon ($n = 6$). Values are given as mean $\pm$ SD. *$p < 0.05$, **$p < 0.01$, **$p < 0.01$ versus control group. DZN: diazinon; CON: control.

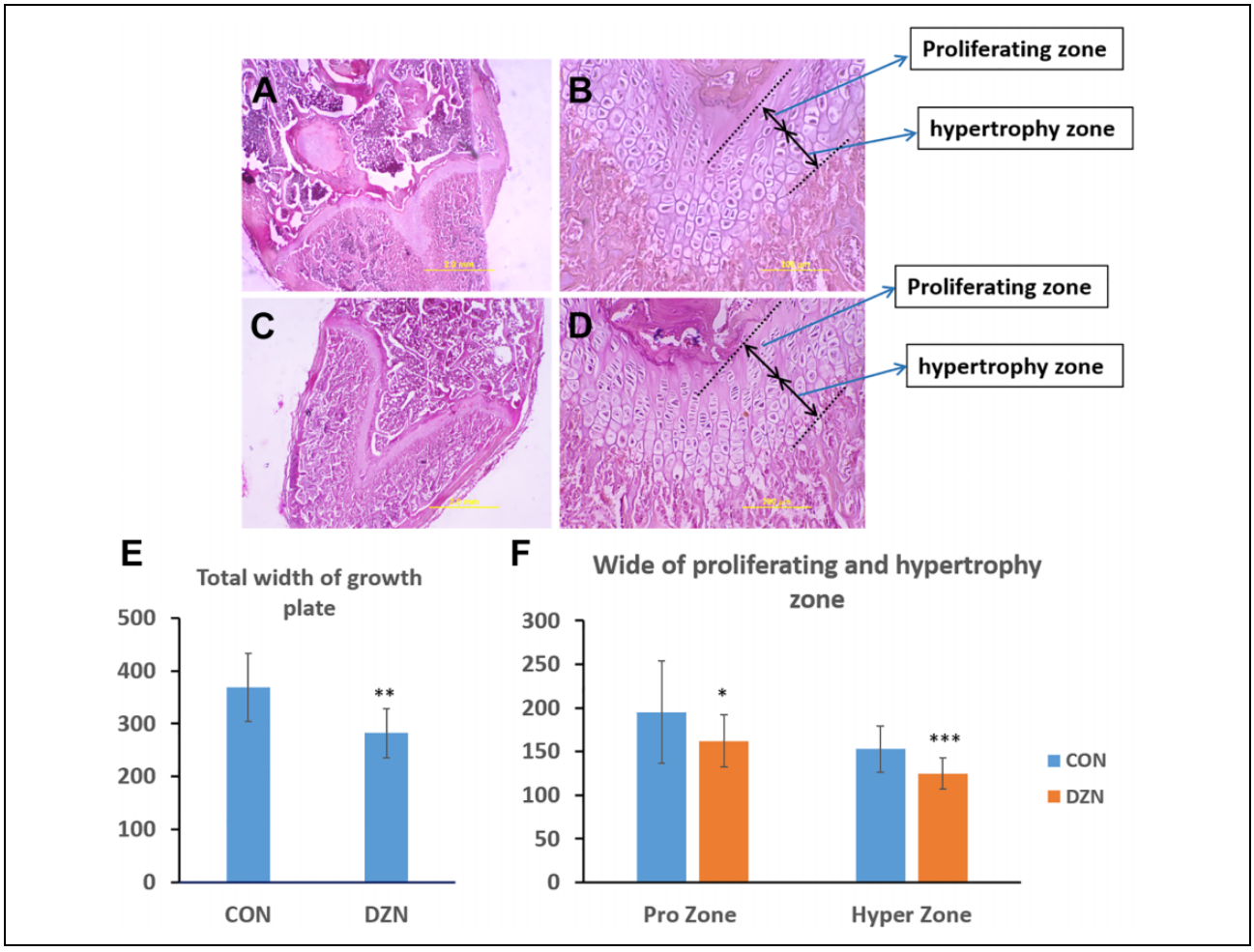

Figure 5. Histological assessment of the effects of DZ on the epiphyseal growth plate.Epiphyseal growth plate cartilage of rat femur stained with H&E (a sample of six selected samples from each group). (A, B): control group. (C, D): DZN group. (A and C) ×100 magnification, (B and D) ×200 magnification. Effect of DZN on the width of E: growth plate, F: proliferation zone, and hypertrophy zone ($n = 6$). Values are given as mean ± SD. *$p < 0.05$, **$p < 0.01$ versus control group. DZN: diazinon; CON: control; H&E: hematoxylin and eosin.

measured. Our results indicated a significant reduction ($p < 0.01$) of overall epiphyseal growth plate width in the DZN group as compared to the control group. Also, a significant decline in the width of the proliferation zone ($p < 0.05$) and that of the hypertrophic zone ($p < 0.05$) in the DZN group was observed in comparison to the control group (Figure 5).

Discussion

Diazinon is a widely used OPs insecticide in agriculture on a variety of fruits, vegetables, nuts, and field crops. This pesticide may affect both humans and animals; therefore, knowledge about the effects of DZN is very important (Yilmaz et al., 2012). This is the first study that investigated the effects of diazinon on bone density following long-term exposure. Previously, it has been shown that diazinon had teratogenic effects on the growth of bone and cartilage of the chick and quail embryos. These effects included reduced growth of the foot and wing, inhibited growth of femur, tibia, and metatarsi as well as twisted claws and reduced bone minerals in the foot (Meneely and Wyttenbach, 1989; Misawa et al., 1982). Also, accidental exposure of diazinon in a family for 2 days caused different long-term effects. In this family, neurotoxicity and endocrine toxicity were reported. That study indicated the diverse effects of DZN on the children's bone, including bone growth retardation, reduced calcification, cyst growth in bones, and pathological fractures (Dahlgren et al., 2004). However, the effects of DZN on bone have not yet been

examined. In this study, for the first time, it was reported that diazinon reduced bone density in both trabecular and cortical bone. Although the exact cellular and molecular mechanisms of these effects have yet to be investigated, our histomorphometric results indicated that DZN damaged the femoral epiphyseal growth plate. Thus, it can be suggested that at least some of the effects of DZN on bone could be attributed to growth plate disruption.

Oral exposure of rats to diazinon indicated its rapid absorption, and about 35% of the oral dose was shown to be systemically bioavailable (Timchalk, 2001). A previous study indicated that after using oral radioactive DZN (10 mg/kg body weight) in rats, this toxicant can be found in bone tissues (0.04 μg/g) (Capps, 1989). Although the concentration of DZN in bone is not high in comparison to other tissues such as the heart (0.1 μg/g), spleen (0.1 μg/g), kidney (0.08 μg/g), and brain (0.09 μg/g) (Capps, 1989), the results of the current study indicated a significant impact of DZN on bone. ACh is expressed in bone tissue and induces proliferation and differentiation of osteoblasts. Inhibition of AChE has been shown to be associated with increased bone mass in humans and animals (Kauschke et al., 2015). We have shown here that the overall effects of DZN on bone is a reduction of BMD; therefore, DZN may affect other direct or indirect regulatory mechanisms of bone turnover that remains to be elucidated further. The bioavalability and benchmark dose of DZN in bone is also not clear and needs to be clarified.

Different zones of growth plate have diverse morphological and biochemical characteristics that are controlled by molecular pathways of growth factors (Lee et al., 2011). In the proliferative regions, chondrocytes appeared flattened and divided to form a column, followed by their enlargement in the hypertrophic zone before degeneration (Ballock and O'Keefe, 2003). In the present study, DZN significantly reduced the width of the proliferation and hypertrophic zone of the growth plate in comparison with the control group. This shrinkage of the growth plate width could be due to the direct effects of DZN on chondrocyte maturity and possible apoptosis, or it could be because of the indirect effects of DZN on growth factors or hormones that affect growth plates such as fibroblast growth factors (Xie et al., 2014) and thyroid hormones (Endo and Kobayashi, 2013; Nazeri et al., 2019). On the other hand, the changes in the growth plate might be one of the reasons for disrupted cortical osteogenesis and calcification processes causing reduced BMD.

In the younger rats, the effects of DZN seemed to be more intense, as the dosage used for adults was lethal for immature rats, and significant reductions were observed in bone density and lamellae following exposure to DZN in immature rats. In rats, the growth of long bones decreases between 7 and 8 months of age (Sengupta, 2013). Thus, although the adult animals (8 weeks) used in the present study might still be in a growing stage, the negative effects of DZN on bones could depend on the growth rate and the ability of the animals to break down and eliminate the toxicant.

In the present study, the effects of different doses of diazinon on femur BMD indicated that at high dosages, it reduced the density of the femoral head, GT, and total bone. The effects of DZN on mitochondria have been previously reported (Ogutcu et al., 2006). It damages the mitochondrial membrane (Miranda et al., 2020), causes vacuolation and swelling in rat liver and heart mitochondria (Ogutcu et al., 2006). Also, it disables p450 cytochromes and microsomes in the human liver and causes changes in the liver enzymes (Ogutcu et al., 2006). Because of the large number of mitochondria in the cells of the proliferative zone (Van der Eerden et al., 2003), it has been suggested that mitochondrial damage in the cells by DZN of this zone can be the reason for the disruption of the proliferating process. Further, in later phases, it may affect the hypertrophy stage. However, this is just an assumption, which remains open for future investigations.

In toxicology research, soft tissues, especially the liver and heart, are mostly the prime targets for investigations. However, due to environmental contaminants, toxic substances in the long term may have wider effects on the body. The results of this study demonstrated that exposure to diazinon reduced bone density. These results lead to many questions regarding the mechanisms of these effects and highlight the need to conducting extensive research in this field. The results of this study could suggest that the presence of this chemical in the environment may be associated with osteoporosis, which is widely observed in human communities.

Authors' note

We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

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Ethical approval

The whole process of the study was carried out in compliance with the guidelines for the care and use of Laboratory Animals published by the National Institutes of Health (NIH Publications No.8023, revised 1978) and was approved by the ethical committee of The Ferdowsi University of Mashhad (Ethics Code: IR.UM.REC.1399.122).

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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