Science of the Total Environment 750 (2021) 141638

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Cadmium exposure triggers osteoporosis in duck *via* P2X7/PI3K/AKT-mediated osteoblast and osteoclast differentiation

Yonggang Ma [1], Di Ran [1], Hongyan Zhao, Ruilong Song, Hui Zou, Jianhong Gu, Yan Yuan, Jianchun Bian, Jiaqiao Zhu *, Zongping Liu *

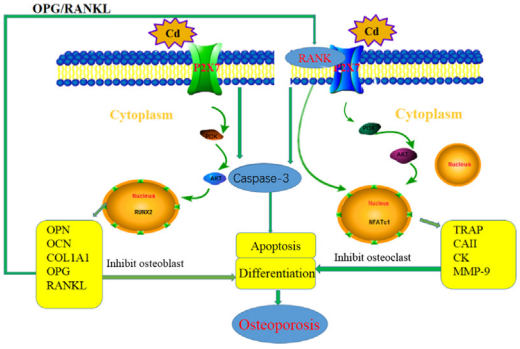
College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, PR China
Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu 225009, PR China
Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education of China, Yangzhou University, Yangzhou, Jiangsu 225009, PR China

HIGHLIGHTS

- Cadmium (Cd) exposure causes osteoporosis in ducks.
- Cd induced osteoporosis is related to defects in osteogenic differentiation.
- Cd exposure decreases protein levels of key osteogenic and bone resorption-related proteins.
- Cd inhibtes the differentiation of osteoclast and osteoblase by inhibiting P2X7/PI3K/AKT pathway.

GRAPHICAL ABSTRACT

Cadmium is a widespread environmental pollutant that accumulates in bone and causes bone damage. Our findings demonstrate that Cd exposure inhibits osteoblast and osteoclast differentiation, and induces apoptosis, finally resulting in osteoporosis. P2X7/PI3K/AKT and OPG/RANKL signaling play an essential role in Cd-induced osteoporosis.



ARTICLE INFO

Article history:
Received 29 May 2020
Received in revised form 8 August 2020
Accepted 9 August 2020
Available online 15 August 2020

Editor: Xinbin Feng

Keywords:
Cadmium
Osteoporosis
P2X7/PI3K/AKT
Duck

ABSTRACT

Cadmium is a common environmental pollutant that accumulates in the bone and kidneys and causes severe health and social problems. However, the effects of Cd on the occurrence of osteoporosis and its mechanism of action in this process are unclear. To test whether Cd-induced osteoporosis is mediated *via* P2X7/PI3K/AKT signaling, duck bone marrow mesenchymal stem cells (BMSCs) and bone marrow macrophage cells (BMMs) were treated with Cd for 5 days, and duck embryos were treated with Cd. Micro-CT analysis indicated that Cd-induced osteoporosis occurs *in vivo*, and histopathology and immunohistochemical analyses also revealed that Cd induced bone damage and the downregulation of osteogenic and bone resorption-related proteins. Cd exposure significantly inhibited the differentiation of BMSCs and BMMs into osteoblasts and osteoclasts *in vitro*, and promoted osteoblast and osteoclast apoptosis. Cd exposure significantly downregulated the P2X7/PI3K/AKT signaling pathway *in vivo* and *in vitro*, and inhibition of this signaling pathway significantly aggravated osteoblast and osteoclast differentiation. Cd exposure also upregulated the OPG/RANKL ratio *in vivo* and *in vitro*, further inhibiting osteoclast differentiation. These results demonstrate that Cd causes osteoporosis in duck by inhibiting P2X7/PI3K/AKT signaling and increasing the OPG/RANKL ratio. These results establish a previously unknown mechanism of Cd-induced osteoporosis.

* Corresponding authors at: College of Veterinary Medicine, Yangzhou University.
E-mail addresses: jqzhu1998@163.com (J. Zhu), liuzongping@yzu.edu.cn (Z. Liu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.scitotenv.2020.141638
0048-9697/

1. Introduction

Cadmium (Cd) is a nonessential heavy metal and a group I carcinogen (Hartwig, 2013) with a long biological half-life of 10–30 years (Sughis et al., 2011). Environmental Cd pollution originates mainly from industrial use and fertilizers (Nazima et al., 2015). Cd cannot be environmentally degraded and enters animal and human bodies through the food chain, and resulting in the accumulation of Cd in ecosystems and in the body (Luo et al., 2017). Cd can accumulate in various organs, such as the bones (He et al., 2020), kidneys (Wang et al., 2016), liver (Rana et al., 2020) and brain (Oboh et al., 2020), causing kidney disease, bone metabolic disease, reproductive diseases and cancer. Numerous studies have shown that poultry are highly susceptible to Cd toxicity (Kar et al., 2018). Recent research suggests that high concentrations of Cd were found in poultry bones in a lead-contaminated goldmine area of Nigeria (Orisakwe et al., 2017), which caused a severe bone metabolic disease in the poultry and subsequent economic damage. In humans, the case of Itai-Itai disease happened in Japan in the 1950s, where people had eaten rice highly contaminated with Cd and developed severe osteoporosis, osteomalacia and fractures. Previous studies have determined that the main mechanism of Cd-induced Itai-Itai disease is kidney damage in the form of glomerular and tubular dysfunction, which also indirectly causes osteoporosis (Nishijo et al., 2017a). However, in order to study the direct effects of Cd exposure on bone, bone morphogenetic protein (BMP) was used to establish a model of induced ectopic osteogenesis that could eliminate the interference of other non-bone factors. This result suggested that Cd had no significant effect on chondrogenesis, but significantly inhibited osteoblast function (Xu et al., 1997). The main cause of Cd-induced osteoporosis has since been determined to be the excessive promotion of osteoclast differentiation (Chen et al., 2017; Miyahara et al., 1992). However, most experimental models of Cd poisoning still fail to replicate osteoporosis, while providing a basis for the injury mechanism in the liver and kidneys. Cd-induced osteoporosis is likely to be a long, dynamic and complex process. Although the mechanism of Cd toxicity is well understood in other organs such as kidney and liver, limited studies exist on bone tissue, especially poultry bones, which can have significant economic impacts. There is currently a lack of knowledge about the toxic effects of cadmium exposure on poultry bones and possible ways to correct them.

Osteoporosis is a “silent disease” that is characterized by low bone mineral density (BMD) and has become a major health problem worldwide resulting in a substantial economic burden on society. The P2X7 receptor is closely associated with osteoporosis (Husted et al., 2013). The P2X7 receptor is an ion channel receptor that provides a calcium-permeable cationic channel (Garcia-Guzman et al., 1996), and is abundantly expressed in osteoblasts, osteocytes and osteoclasts (Volonté et al., 2016). Therefore, P2X7 may play an essential role in regulating the balance of osteoclast resorption and osteoblast bone formation. Once this balance is broken, it can result in bone metabolic disease. The activation of P2X7 induces high intracellular Ca^{2+} concentrations and activates a series of signaling pathways including MAPK, NF-κB and PI3K/AKT (Genetos et al., 2011; Grol et al., 2012; Liu et al., 2008). Meanwhile, the activation of P2X7 promotes osteoblast and osteoclast function, and the accelerated transcription of the osteogenic transcription factor RUNX2 and osteoclast differentiation factor NFATC1 (Korcok et al., 2004; Panupinthu et al., 2008). Moreover, the absence of P2X7 inhibited osteoclast differentiation (Ma et al., 2019), but the knockout of P2X7 showed no effect on osteoclast function in an animal model (Gartland et al., 2003b). Therefore, the effect of P2X7 is controversial in the regulation of osteoclast function and requires further study. It is also unclear whether Cd, as an extracellular molecule, can affect P2X7 activation or P2X7-mediated downstream signaling. Therefore, a thorough inquiry into the role of P2X7 during Cd-induced bone tissue damage is urgently needed.

The aim of this study is to evaluate the mechanism of Cd-induced bone damage *in vivo* and *in vitro*. P2X7 is a key molecule for maintaining bone metabolism, and bone dysfunction is a manifestation of Cd poisoning. This study explores the direct effect of Cd exposure on the structure of duck bones through the P2X7-mediated signaling axis, providing a new understanding of Cd-induced bone metabolic disease.

2. Materials and methods

2.1. Experimental animals

Animal experiments were approved by the Institutional Animal Care and Use Committee of Yangzhou University. Healthy 2-day-old Gaoyou duck embryos were obtained from the Waterfowl Gene Pool of Quality Resources. Two-day-old duck embryos were randomly assigned to two groups ($n = 6$) as follows: the control group and the Cd exposure group. The control group was inoculated in the yolk sac with saline, while the Cd group was inoculated in the yolk sac with 1 μM $CdCl_2$ for each gram of embryo. After the duck embryo was shelled, ducks were free to eat and drink *ad libitum* for 14 days. All ducks were sacrificed *via* euthanasia. Bone tissue was carefully separated and soaked in 4% paraformaldehyde before being stored at −80 °C for subsequent experiments.

2.2. Reagents

$CdCl_2$ was purchased from Sigma Company, USA, and the inhibitor A438079 was obtained from Abcam, USA. Other all reagents were purchased in China (Bibang Biology, Yangzhou, China).

2.3. Cell isolation and culture

One-day-old ducks were euthanized and sterilized with 75% ethanol, after which bone marrow mesenchymal stem cells (BMSCs) were isolated from the femurs. Cells were cultured for a period of 4–5 days in α-MEM at 37 °C under 5% CO_2, and cells were passaged when adherent cells reached a density of 80%–90%. BMSCs were used at passages three to six in the following experiments. Bone marrow macrophages (BMMs) were isolated from duck femurs and extracted cells were culture in α-MEM containing 10% FBS in a humidified incubator 37 °C and 5% CO_2. BMMs were used for differentiation into osteoclasts.

2.4. Alkaline phosphatase (ALP) and Alizarin red staining

Duck BMSCs were cultured with different concentrations (0, 0.1, and 1 μM) of Cd for 4 days; duck BMSCs were pretreated with A438079 (10 μM), BKM120 (1 μM), and MK2206 (1 μM) for 30 min, then treated with Cd (1 μM). After osteogenic induction, cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min, and stained with the ALP staining kit (Beyotime, China) or Alizarin red staining kit (Cyagen, China). Images were collected with an Observer microscope (Leica, Germany).

2.5. Tartrate resistant acid phosphatase (TRAP) staining and bone resorption

Duck BMMs were cultured with different concentrations (0, 0.1, and 1 μM) of Cd for 4 days in α-MEM. Duck BMMs were pretreated with A438079 (10 μM), BKM120 (1 μM), and MK2206 (1 μM) for 30 min, then treated with Cd (1 μM) for 5 days. To identify osteoclasts, the cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. The cells were then stained using the TRAP staining kit (Sigma, St. Louis, USA) according to the manufacturer's instructions. Osteoclast numbers were observed by microscopy (Leica, Germany). Duck BMMs were seeded on Corning Osteo-assay surface 96-well plates (Coring, USA). After the incubation period, cells were washed with PBS 5–6 times. Then, the resorption area was observed with microscopy.

2.6. Western blot analysis

Duck BMSCs and BMMs were seeded in 12-well plates and treated with 0, 0.1, and 1 μM of Cd for 4 days; duck BMSCs and BMMs were pretreated with A438079 (10 μM), BKM120 (1 μM), and MK2206 (1 μM) for 30 min, then treated with Cd (1 μM) for 4 days. Duck embryos were inoculated with 1 μM in the yolk sac for each gram of embryo. Bone tissue (10 mg) was homogenized in a 250 μL centrifuge tube containing RIPA buffer (NCM, China) for the rapid preparation system of biological samples (Life Real, China). Cells were washed twice with cold PBS, lysed in RIPA containing a proteinase inhibitor cocktail buffer on ice for 30 min, and then centrifuged at 12,000 ×*g* for 10 min to precipitate cell and bone tissue debris. The protein concentration was determined using the BCA protein assay kit (NCM Biotech, China). Equal amounts of protein were separated on a SDS-polyacrylamide gel. The proteins in the gel were transferred onto PVDF membranes (Millipore, USA). After blocking with 5% skim milk in TBST for 2 h, the membranes were incubated first with primary for 12 h at 4 °C and then with appropriate secondary antibodies for 2 h at RT. The following antibodies were used: OPN, OCN, OPG, RANKL, RUNX2, COL1A1 (1:500, ABclonal, China); TRAP, CAII, CK, NFATc1, c-fos (1:1000, Abcam, USA); Bax, Bcl2, caspase-3, LC3, P62, β-actin (1:1000, CST, USA). The membranes were imaged with chemiluminescence (NCM, China). Data quantification analyses were performed using ImageJ software (Image, Inc., USA).

2.7. Apoptosis assay

Duck BMCSs and BMMs were seeded in 6-well plates for 4 days, and after induction cells were treated with 1 μM of Cd for 12 h. Then, flow cytometry was carried out to analyze the effect of Cd. Cells were collected and stained with the Annexin V-FITC/PI apoptosis kit (Vazyme, China). Following isolation cells were immediately analyzed on a flow cytometer (BD, USA).

2.8. Cellular viability and ALP and ATP detection

Duck BMSCs and BMMs were treated with 0, 0.1, and 1 μM Cd for 4 days. The viability of BMSCs and BMMs was assayed by cell counting kit-8 (CCK-8) assay (Vazyme, China). ATP and ALP content was detected by the Duck ATP detection kit (Mlbio, China) and the duck ALP kit (Mlbio, China).

2.9. Microcomputed tomography (micro-CT) analysis

$CdCl_2$ was inoculated in the yolk sac with 1 μM Cd for each gram of embryo. After the duck embryo was shelled, ducks were free to eat and drink *ad libitum* for 14 days. Micro-CT images (Skyscan1174 X-Ray Microtomograph, Bruker) were used to evaluate the effect of Cd on duck bone microstructure. The tibia was scanned with the following parameters: voltage, 50 kW; electric current, 800 μA; resolution, 12 μm per pixel. After scanning, the regions of interest (ROIs) were selected and the N-Reco software was used for 3D image reconstruction. Bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and the structural model index (SMI) were analyzed by CT-AN software.

2.10. Histopathological assessment

Duck bone tissue was soaked in Perenyi's decalcifying solution for 2 weeks, and dehydrated using ethanol gradients. Then, the bone tissue was embedded in paraffin, cut into 5-μm thick sections, and stained with hematoxylin and eosin (H&E) for microscopic examination. Histopathological analysis was performed by a pathologist.

2.11. Histochemistry and immunohistochemistry

Tartrate resistant acid phosphatase (TRAP) staining was used to analyze duck tibia osteoclast formation. The method was performed as previously described (Chen et al., 2017). The TRAP-positive area was measured with Image-pro Plus 6.0 software (Image, Inc., USA).

Immunohistochemistry was used to evaluate the expression of key bone tissue proteins. The technique was based on our previous study (He et al., 2020). All primary antibodies (OPN, OCN, OPG, RANKL) and secondary antibodies were obtained from Wuhan Servicebio, and the dilution was 1:200. The results were analyzed by immunohistochemical average optical density value, and the Image-pro Plus 6.0 software was used to select the brown–yellow color as the unified standard for judging the positive areas of all photos, after which the cumulative optical density value (IOD) and the pixel AREA (AREA) of the tissue were obtained by analyzing each photo. The average optical density (AO value) was calculated as AO = IOD / AREA. As the AO value grows higher, so does the positive expression level.

2.12. Statistical analysis

Statistical analysis of all data was performed using GraphPad Prism 7 (GraphPad Software, Inc., USA). Each experiment was repeated at least three times. All quantitative data are represented as means ± SD. Asterisks (*) indicate statistical significance compared with the control group, $^{*}P < .05$, $^{**}P < .01$.

3. Results

3.1. The effect of Cd on BMSCs and BMMs into osteoblast and osteoclast differentiation

To investigate the effect of Cd exposure on osteoporosis in ducks at the molecular level, the differentiation indices of duck BMSCs and BMMs were detected. Firstly, after exposure to Cd (0, 0.1, and 1 μM) for 12 h. The viability of BMSCs was not significantly changed with increasing concentrations of Cd as determined by CCK-8 assay (Fig. 1a), but there was a clear increase in BMM viability with 0.1 μM Cd, and no change with 1 μM (Fig. 1b). After Cd exposure for 4 days, the results show that Cd significantly inhibited osteoblast differentiation from BMSCs as determined by ALP staining and ALP activity (Fig. 1c,e). Alizarin red staining also suggested that Cd suppressed the mineralization potential of BMSCs as mineralization nodules significantly decreased in the late stage of osteogenic differentiation (Fig. 1d). The expression of osteogenic-related proteins also significantly decreased with increasing concentration of Cd as determined by Western blotting, including osteocalcin (OCN), osteopontin (OPN), osteoprotegerin (OPG), runt-related transcription factor 2 (RUNX2), collagen type 1 alpha 1 (COL1A1) and RANKL (Fig. 1g). These results demonstrate that Cd suppresses the osteogenic differentiation of duck BMSCs.

Interestingly, we found that the ratios of OPG/RANKL obviously increased as detected by Western blotting (Fig. 1f). However, in our study duck BMMs were treated with Cd for 5 days, and TRAP staining shown that Cd significantly inhibited osteoclast formation (Fig. 1h), while bone resorption was suppressed in a dose-dependent manner (Fig. 1i). Western blotting suggested that the expression of osteoclast formation- and differentiation-related proteins significantly decreased with increasing of Cd exposure concentrations, including MMP-9, CA, CKII, TRAP, c-fos and NFATc1 (Fig. 1j). Therefore, the above results demonstrate that Cd exposure triggers a significant inhibition of osteoclast differentiation.

3.2. The effect of Cd on P2X7/PI3K/AKT signaling

To thoroughly assess the effect of Cd on BMCSs and BMMs in osteoblast and osteoclast differentiation, we examined the intracellular ATP

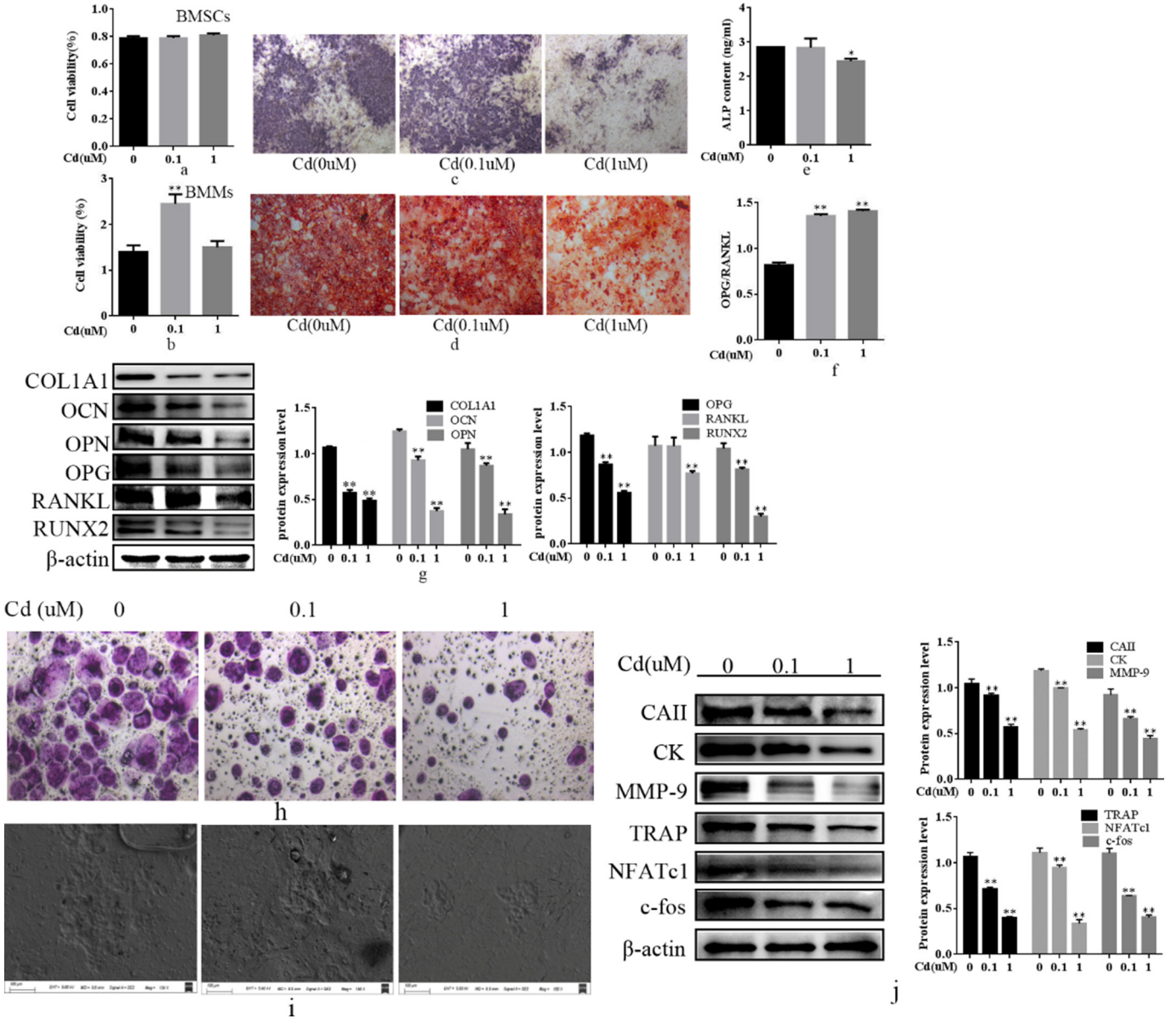


Fig. 1. The effect of Cd on BMSCs and BMMs in osteoblast and osteoclast differentiation. (a, b) Duck BMSC and BMM viability measured by CCK-8. (c) ALP staining. (d) Alizarin Red staining. (e) ALP activity as measured by the ALP kit. (f) The OPG/RANKL ratio. (g) Osteogenesis-related protein expression. (h) TRAP staining. (i) Bone resorption. (j) Bone resorption related protein expression. Values are expressed as the mean ± SD of three individual experiments, Significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

levels and ATP-mediated related signaling molecules. ATP levels gradually decreased in the Cd-exposed duck BMSCs and BMMs during osteoblast and osteoclast differentiation (Fig. 2a). Meanwhile, the expression of P2X7 was clearly decreased after Cd exposure for 12 h in osteoblasts and osteoclasts (Fig. 2b). The phosphorylation level of PI3K/AKT was also reduced as Cd concentrations increased (Fig. 2b). These results indicate that Cd inhibited the P2X7/PI3K/AKT signaling pathway in BMSCs and BMMs during osteoblast and osteoclast differentiation.

3.3. The effect of P2X7/PI3K/AKT signaling on Cd induced osteoblast and osteoclast differentiation

We used a P2X7/PI3K/AKT signal inhibitor to measure the effect of P2X7/PI3K/AKT signaling on Cd-induced osteoblast and osteoclast differentiation. ALP and Alizarin red staining showed that pathway inhibitors A438079, BKM120, and MK2206 significantly suppressed Cd-induced osteoblast differentiation compared with the Cd-alone group (Fig. 3a). Western blots suggested that the expression of osteogenesis-related proteins decreased more after inhibitor exposure compared with Cd alone (Fig. 3a). However, TRAP staining and bone resorption results also showed that the TRAP-positive multinuclear osteoclast number and bone resorption pit were radically reduced compared with Cd when P2X7/PI3K/AKT signaling was blocked (Fig. 3b). Osteoclast formation and differentiation was also significantly reduced compared with the Cd group (Fig. 3b). These results demonstrate that blocking P2X7/PI3K/AKT signaling aggravated Cd-induced osteoblast and osteoclast differentiation.

3.4. The effect of Cd on osteoblast and osteoclast apoptosis

Flow cytometry results showed that Cd induced the apoptosis of osteoblasts and osteoclasts derived from BMSCs and BMMs (Fig. 4a).

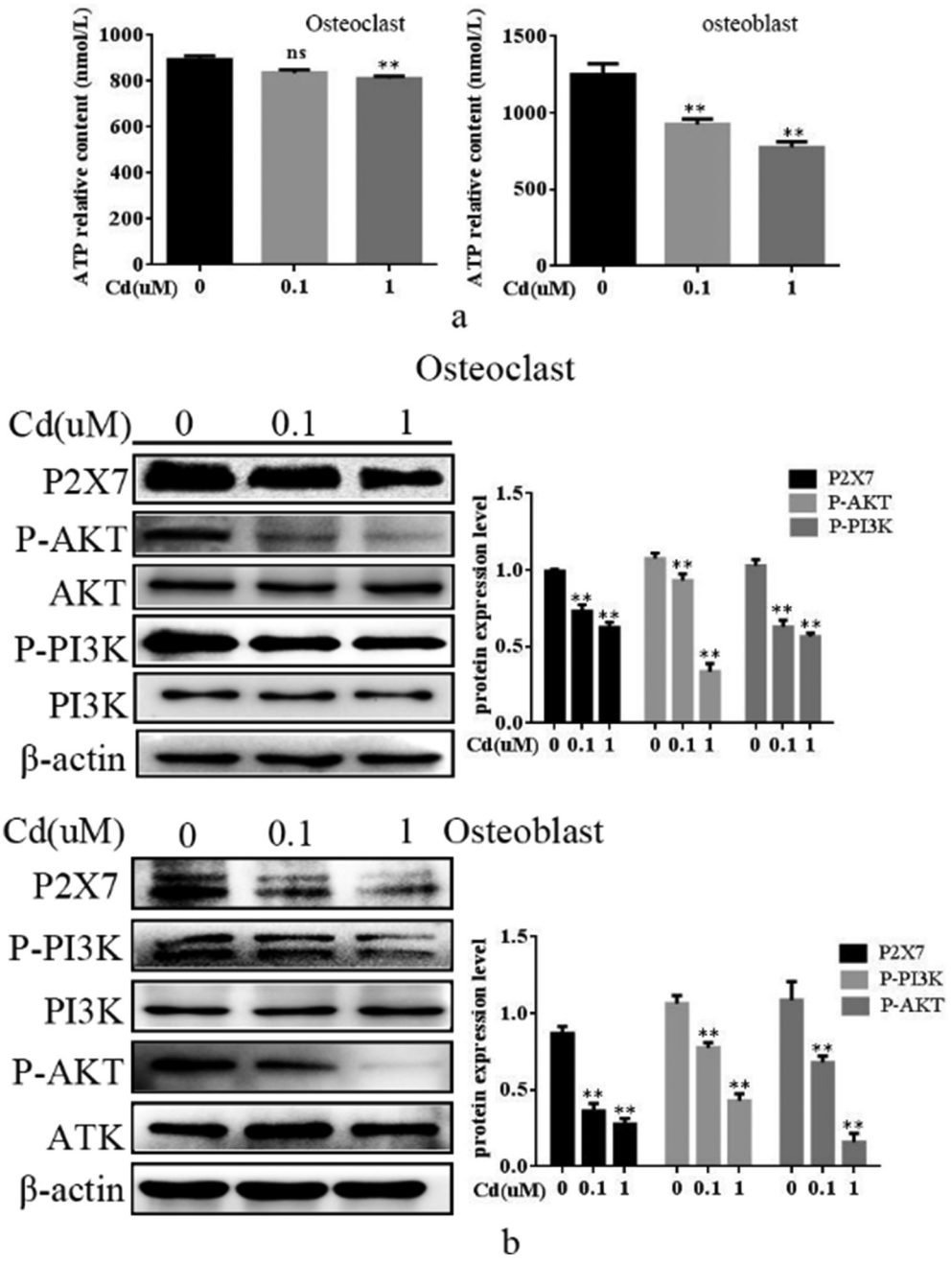


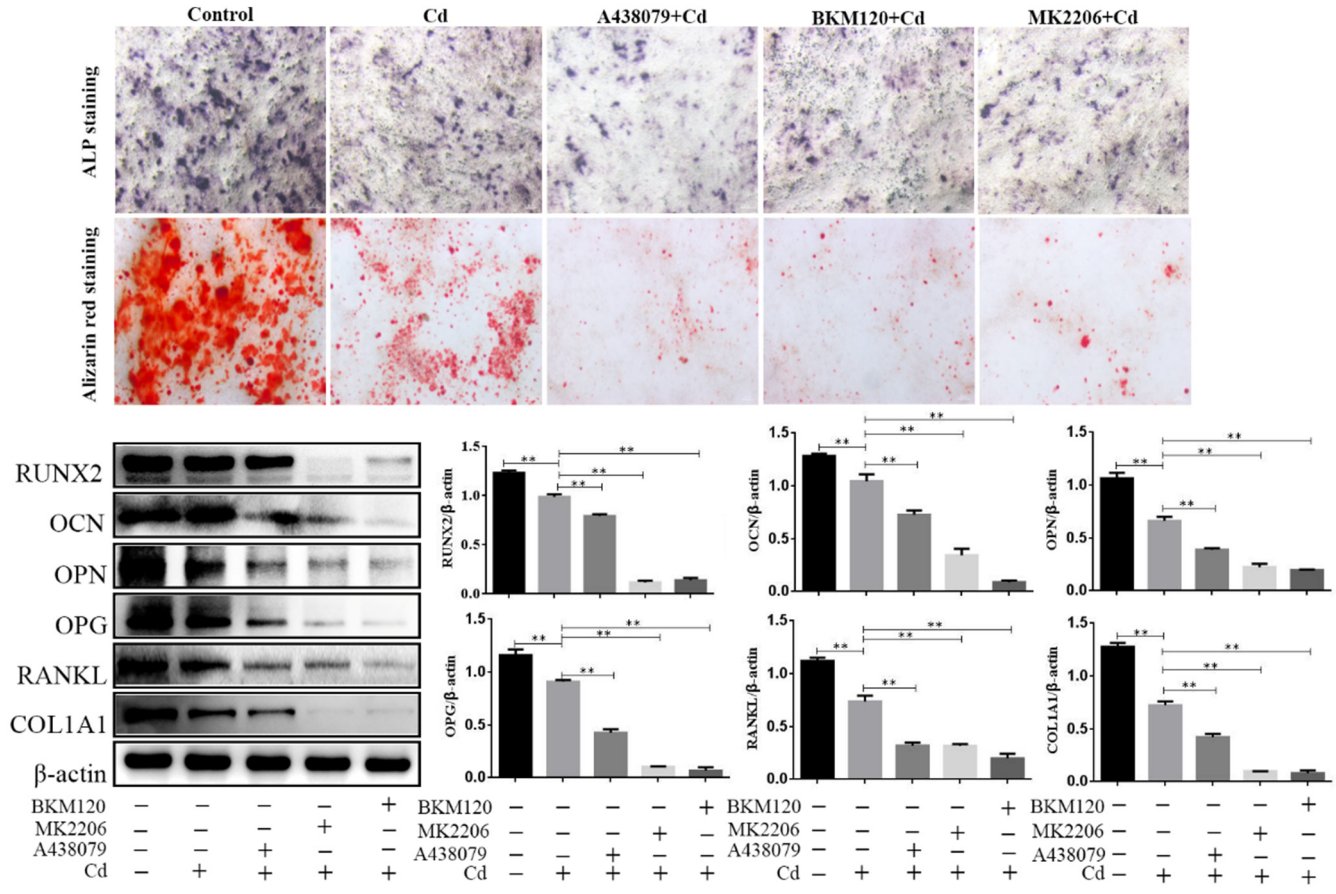
Fig. 2. The effect of Cd on P2X7/PI3K/AKT signaling. (a) ATP content as measured by the ATP detection kit. (b) The expression of P2X7 and the phosphorylation of PI3K/AKT as measured by Western blotting. Values are expressed as the mean ± SD of three individual experiments, significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

Western blotting suggested that the expression of Bax and cleaved caspase-3 also significantly increased with the increasing Cd concentration, especially at 1 μM (Fig. 4b). Cd can therefore induce the apoptosis of osteoblasts and osteoclasts.

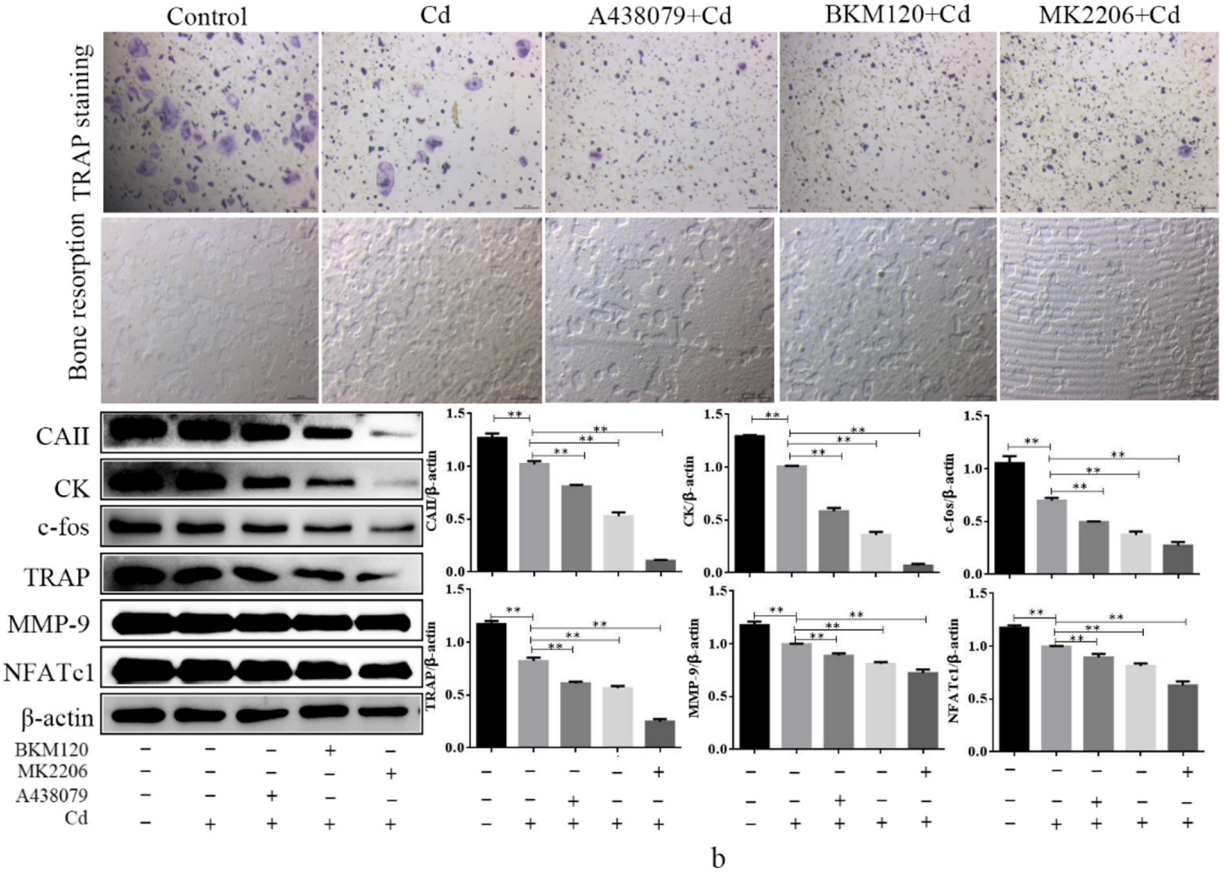
3.5. The effect of Cd on body weight and bone microarchitectural and bone histopathological indices

To determine the effect of Cd on bone damage, the body weights of ducks were measured, and no significant difference was observed between the Cd group and the control group (Supplementary 1). No ducks died during the experiments. We also analyzed tibia microstructural parameters using Micro-CT. Three-dimensional image analysis revealed that BV/TV, TB·N and BMD were significantly reduced after treatment with Cd, and Tb.Sp was significantly increased in the Cd-treatment group compared with the control group. No significant differences were found in Tb. Th and SMI between groups (Fig. 5a). These results indicated that Cd induces osteoporosis.

To further investigate bone injuries induced by Cd, Fig. 5b shows that the outer layer of bone tissue is a ring of cancellous bone (black arrow) with a large mass of bone marrow cells (red arrow). However, a large mass of fat cells (yellow arrow) appears in the middle after Cd treatment. On the contrary, in the control group the outer layer of bone tissue is a ring of cancellous bone (black arrow) with a large number of chondrocytes or other bone cells (red arrow) in the middle. Together, these results demonstrate that bone tissues were injured in response


Control
Cd
A438079+Cd
BKM120+Cd
MK2206+Cd
ALP staining
Alizarin red staining
RUNX2
OCN
OPN
OPG
RANKL
COL1A1
β-actin
RUNX2/β-actin
OCN/β-actin
OPN/β-actin
OPG/β-actin
RANKL/β-actin
COL1A1/β-actin
BKM120
MK2206
A438079
Cd

a


Control
Cd
A438079+Cd
BKM120+Cd
MK2206+Cd
TRAP staining
Bone resorption
CAII
CK
c-fos
TRAP
MMP-9
NFATc1
β-actin
CAII/β-actin
CK/β-actin
c-fos/β-actin
TRAP/β-actin
MMP-9/β-actin
NFATc1/β-actin
BKM120
MK2206
A438079
Cd

b

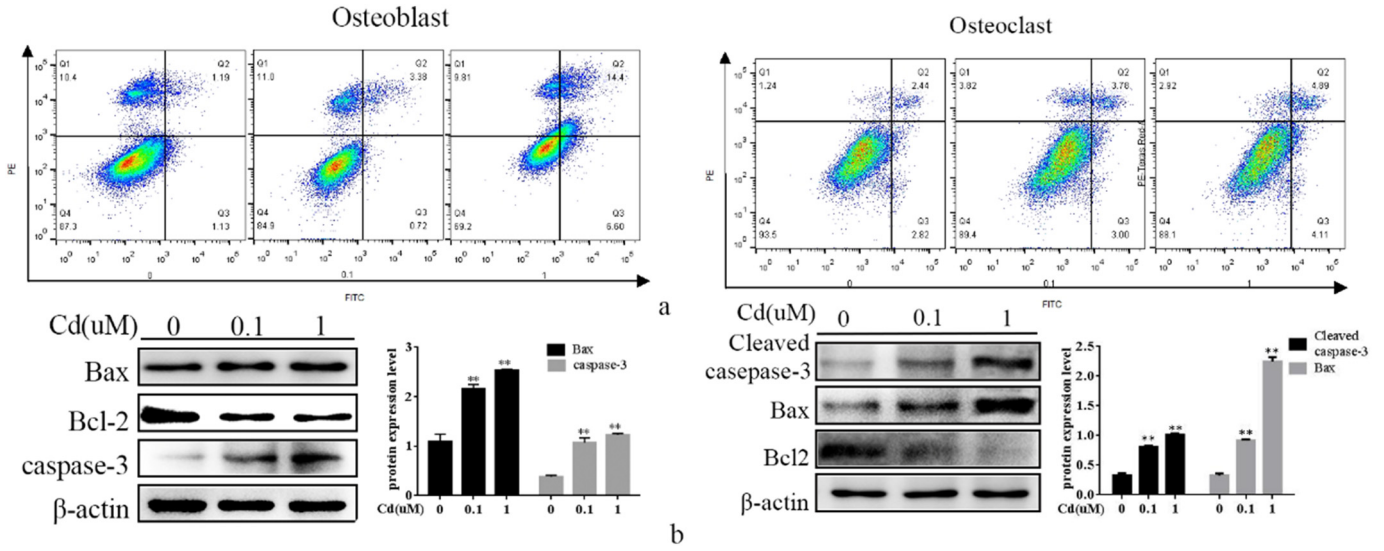


Fig. 4. The effect of Cd on osteoblast and osteoclast apoptosis. OB and OC were treated with Cd for 12 h. (a) Apoptosis rate as measured by flow cytometry. (b) Bax and cleaved caspase-3 protein expression. Values are expressed as the mean ± SD of three individual experiments, Significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

to Cd treatment, and a large number of bone cells disappeared and were replaced by fat cells.

3.6. The effect of Cd on the expression of key bone tissue proteins

As shown in Fig. 6a, the number of osteoclasts was significantly decreased in Cd-treated ducks. It is generally known that key osteogenesis proteins are abundant in osteoblasts and other bone cells, except for osteoclasts. Immunohistochemistry staining results showed that the positive expression of key osteogenesis proteins such as OPN, OCN, COL1A1, RANKL and RANK were significantly reduced in the Cd-treated group, while the expression of OPG was significantly increased, and positive TRAP-expressing cells were decreased after Cd treatment (Fig. 6a). Bone tissue-related proteins were measured by Western blotting, as shown in Fig. 6b, and in the Cd-treated group the expression levels of COL1A1, OPN, OCN, RANKL, TRAP and RUNX2 were reduced compared with the control group. OPG showed no obvious difference between Cd and control groups, but the ratio of OPG/RANKL was significantly increased. In addition, key apoptosis proteins were also analyzed using immunohistochemistry and Western blotting. The expression of apoptosis-related protein (Bax, cleaved caspase-3) was increased in the Cd-treated group (Fig. 6c). Therefore, these results reveal that Cd suppresses the expression of key osteogenesis proteins, increases the ratio of OPG/RANKL and promotes bone tissue apoptosis.

3.7. The effect of Cd on P2X7/PI3K/AKT signaling in bone tissue

According to microarchitectural and bone histopathological indices, Cd damages bone tissues and induces osteoporosis. Therefore, we asked whether P2X7/PI3K/AKT signaling plays a key role in Cd-induced osteoporosis. We examined ATP levels and expression of key P2X7/PI3K/AKT signaling proteins with an ATP detection kit and Western blotting. In bone tissue, ATP levels were decreased in the Cd-treated group (Fig. 7a), and the expression of P2X7 also was significantly reduced while the phosphorylation levels of PI3K and AKT were significantly decreased in the Cd-exposed group compared with the control group (Fig. 7b). These results indicated that Cd exposure inhibited P2X7/PI3K/AKT signaling in bone tissue.

4. Discussion

Cd is a persistent environmental and occupational pollutant that can enter human and animal organs through a variety of pathways (Duranova et al., 2014; Radwan and Salama, 2006). Increasing research demonstrates that Cd exposure causes many diseases, including bone damage and renal dysfunction (Fan et al., 2018), that lead to a series of social problems related to human and animal health. Previous studies indicated that Cd induced osteoclast differentiation and increased osteoblast apoptosis and autophagy (Chen et al., 2013; Liu et al., 2016). This study investigated how Cd exposure affects osteoporosis in ducks. The results revealed that Cd suppresses the differentiation of osteoblasts and osteoclasts, stimulates osteoblast and osteoclast apoptosis, and causes osteoporosis. However, P2X7/PI3K/AKT signaling also plays a pivotal role in Cd-induced osteoporosis.

After Cd enters the body, kidneys and bone are the main target organs, with approximately 50%–80% of Cd accumulating in bone and kidney, which causes severe glomerular and tubular dysfunction and finally results in osteoporosis (Nishijo et al., 2017b; Zhang et al., 2012). Hence, kidney damage may be the main cause of Cd-induced osteoporosis. In this study, we explored the direct effect of Cd on bone structures and differentiation. Firstly, Cd had no significant influence on the weight of ducks, which was consistent with an earlier report (Chen et al., 2013). Bone microstructure and histopathology were observed, and after Cd exposure BV/TV, TB. N and BMD were significantly reduced; also, although Tb.Th and SMI showed no significant difference, they were slightly lower than those in control group, which may be related to Cd exposure time. A large number of bone cells disappeared and were replaced by fat cells, which has been demonstrated in many studies (Knani et al., 2019), since Cd suppressed the differentiation of BMSCs to osteoblasts, and increased differentiation into adipocytes (Rodríguez and Mandalunis, 2016). In addition, TRAP staining showed that osteoclast numbers were also decreased in bone tissue. Therefore, bone damage is directly related to Cd exposure.

Fig. 3. The effect of P2X7/PI3K/AKT signaling on Cd induced osteoblast and osteoclast differentiation. (a) ALP staining, Alizarin Red staining and Western blotting was used to analyze the effect of P2X7/PI3K/AKT signaling on Cd-induced osteoclast and osteoblast differentiation. Values are expressed as the mean ± SD of three individual experiments, significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

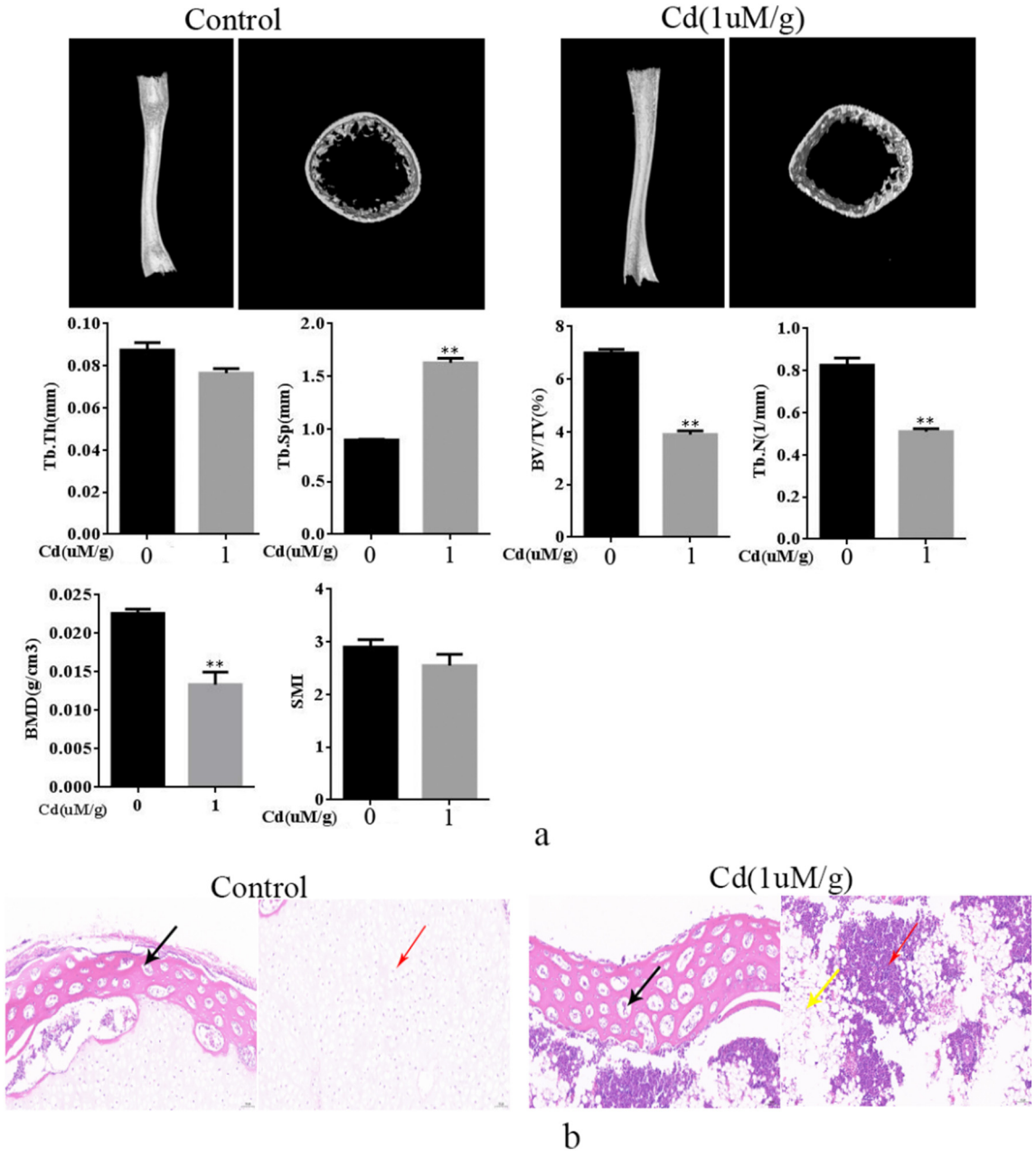


Fig. 5. The effect of Cd on body weight and bone microarchitectural and bone histopathological indices. (a) Tibia analyzed by Micro CT. (b) Bone tissue damage as measured by H&E staining. Values are expressed as the mean ± SD of three individual experiments, significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

Cd-induced osteoporosis due to increased differentiation and bone resorption of osteoclasts can result in disruption of bone metabolism (Chen et al., 2017; Wang et al., 2014). However, in our study, after BMMs were treated with Cd (0.1–10 μM), TRAP-positive multinuclear osteoclast numbers were significantly decreased, and the same Cd concentration also inhibited the differentiation of osteoblasts and prevented bone mineralization. *In vivo* experiments also indicated that the expression of key osteogenesis related proteins and bone resorption protein were significantly reduced after Cd exposure. Although bone resorption functions are reduced *in vivo* and *in vitro*, the ducks still present with osteoporosis. This may be due to the dual effect of Cd on osteoclasts. Research suggested that low-dose Cd exposure can increase osteoclast function in the presence of osteoblasts *in vitro*; meanwhile, the expression of RANKL was significantly increased, but did not inhibit osteoblast function (Chen et al., 2012). However, we also reported that Cd exposure did not affect ALP activity in bone tissue, but increased the ratio of RANKL/OPG (He et al., 2020). RANKL plays an essential role in osteoclastogenesis (Soysa and Alles, 2016), osteoprotegerin (OPG) as a decoy receptor, which inhibits osteoclast differentiation (Ma et al., 2019). Therefore, osteoclast activity is increased *via* regulating the RANKL/OPG ratio because of Cd exposure. In contrast, in our study Cd exposure upregulated the ratio of OPG/RANKL, which inhibited osteoclast differentiation. In previous studies Cd promoted osteoclast differentiation in the presence of RANKL, but these results exclude the role of RANKL. Duck BMMs were treated with Cd without RANKL to directly explore the effect of Cd on osteoclasts because RANKL secreted by osteoblast plays an important role in osteoclastogenesis. Osteoclast bone resorption is inhibited by Cd without RANKL (Iwami and Moriyama, 1993). Our results indicated that Cd plays a dual role in regulating osteoclast function: while Cd inhibits osteoclast function, it also induces

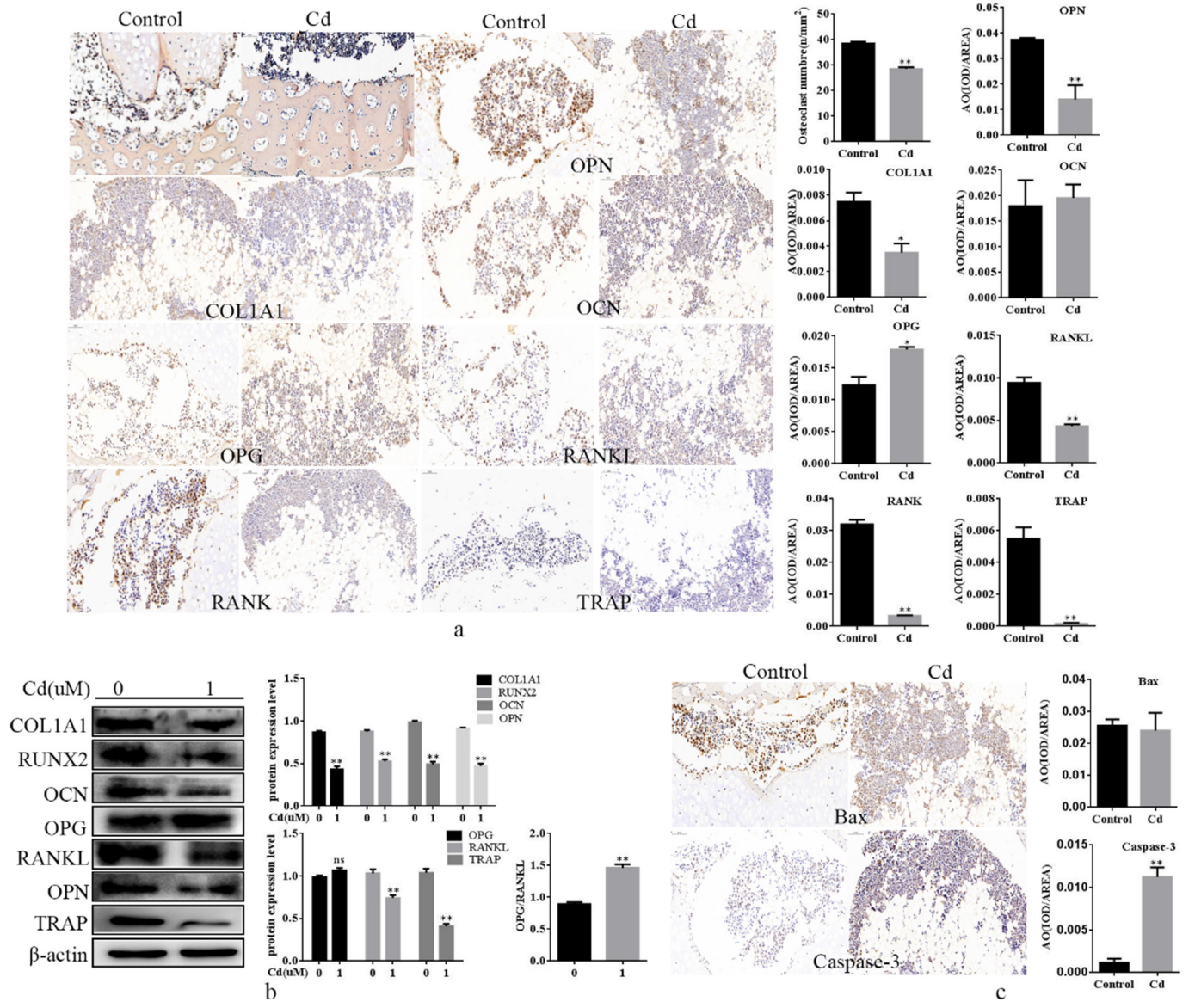


Fig. 6. The effect of Cd on the expression of bone tissue key protein. (a) Osteogenesis proteins as measured by immunohistochemistry. (b) Osteogenesis- and osteoclast-related protein expression as measured by western blotting. (c) Bax and cleaved caspase-3 protein expression. Values are expressed as the mean ± SD of three individual experiments, significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

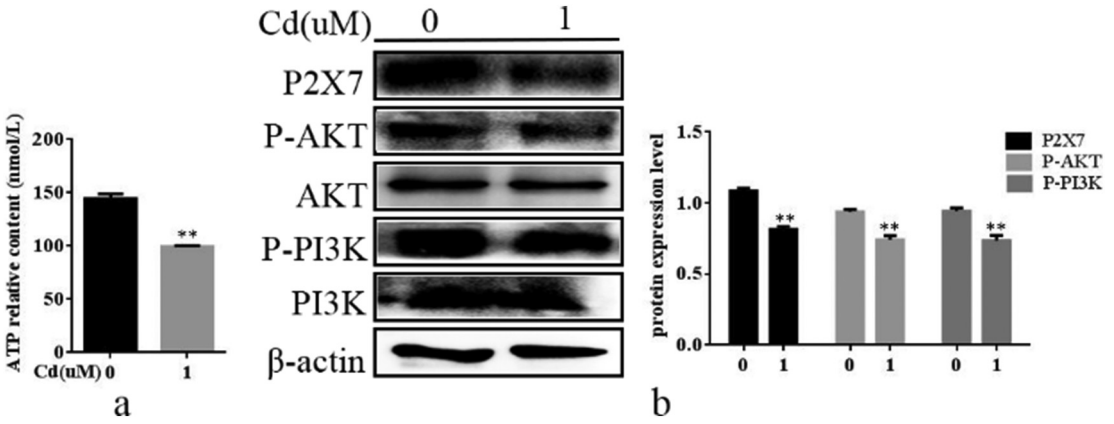


Fig. 7. The effect of Cd on P2X7/PI3K/AKT signaling in bone tissue. (a) Bone tissue ATP as measured by the ATP detection kit. (b) Expression of P2X7 and phosphorylation of PI3K/AKT as measured by Western blotting. Values are expressed as the mean ± SD of three individual experiments, significant differences between Cd-treated groups and control groups are denoted as follows: ** $P < .01$.

osteoporosis. The main reason is that osteogenesis plays a critical role in Cd-induced osteoporosis. In this study, low-dose Cd exposure downregulated osteogenic differentiation genes, but increased the gene expression of RANKL and decreased gene expression of OPG while the TRAP-positive osteoclast number increased. However, rats present no osteoporosis or kidney damage from Cd exposure (Lv et al., 2019). The main reason is that Cd might act independently on bone. A modest increase in osteoclasts is not the direct cause of cadmium-induced osteoporosis, since osteogenic-related cells are highly susceptible to Cd-mediated osteoporosis. In contrast, in this study Cd exposure significantly decreased the expression of osteogenesis proteins (OPN, OCN, COL1A1, RUNX2), the expression of OPG and RANKL were reduced, and the expression of bone resorption proteins and osteoclast numbers were all decreased in ducks presenting with osteoporosis. Therefore, osteogenesis plays a leading role in Cd-induced osteoporosis.

To further explore the mechanisms involved in Cd-induced osteoporosis, we focused on apoptosis, which plays an important role in maintaining many cellular functions (Heitzer et al., 2020). Several studies have focused on the apoptosis of osteoblasts induced by Cd (Arbon et al., 2012; Hu et al., 2015), but it has not been reported that Cd induces apoptosis in osteoclasts. In previous studies, Cd exposure caused osteoblast apoptosis by a series of signaling pathways in rat or human osteoblast-like cell lines (Hu et al., 2015). In this study, Cd exposure-increased apoptosis was observed in osteoblasts derived from duck BMSCs. Although Cd-induced osteoclast apoptosis had not been reported, we found that Cd concentrations from (0.1–1.0 μM) significantly increased the osteoclast apoptosis rate. In summary, apoptosis is involved in Cd-induced osteoporosis.

The P2X7 receptor is part of a class of unique ligand-gated nonselective channels (Garré et al., 2020) that are broadly expressed in osteoblasts and osteoclasts (Dong et al., 2020; Zhang et al., 2019). ATP is an essential extracellular signaling molecule that is involved in many physiological processes such as autophagy (Chen et al., 2020b), apoptosis (Chen et al., 2020a) and cell differentiation (Xiong et al., 2017). Activation of P2X7 by ATP plays a significant role in the regulation of osteoblast and osteoclast function (Gartland et al., 2003a; Orriss et al., 2012; Sun et al., 2013). In addition, P2X7 also mediates many pathological processes, especially bone-related diseases (Gu et al., 2001). However, current research has found contradiction in the effect of P2X7 regulating osteoblast and osteoclast function and in osteoporosis. Therefore, the effect of P2X7 on pathological conditions should be further explored, as P2X7 may play an important role in Cd-induced osteoporosis. In this study, the expression of P2X7 was significantly decreased after Cd exposure, and concomitantly inhibited osteoblast and osteoclast differentiation. The expression of P2X7 was also significantly reduced in Cd-induced osteoporosis. We also found that A438079, a P2X7 inhibitor, further suppressed osteoblast and osteoclast differentiation after Cd exposure. Consistent with our results, P2X7 antagonizes the formation and function of human osteoclasts *in vitro* (Agrawal et al., 2010), and P2X7 induces osteogenic differentiation and mineralization of postmenopausal bone marrow-derived mesenchymal stem cells (Noronha-Matos et al., 2014). Consequently, these results indicated that Cd causes osteoporosis *via* regulating P2X7. Previous results showed that the activation of P2X7 promoted PI3K/AKT signaling, and mediated the growth and metastasis of osteosarcoma cells (Zhang et al., 2019). PI3K/AKT signaling also plays an important role in RANKL-induced osteoclast formation (Lu et al., 2020), and the activation of PI3K/AKT signaling also promotes the expression of RANKL in osteoblasts (Sun et al., 2020) and increases osteoclast differentiation. Consistent with those results, during osteoblast and osteoclast differentiation Cd exposure significantly suppressed the PI3K/AKT signaling pathway, and inhibition of PI3K/AKT signaling further suppressed Cd-induced osteoblast and osteoclast differentiation. In addition, in Cd-induced osteoporosis, Cd exposure also inhibited PI3K/AKT signaling. Therefore, P2X7/PI3K/AKT signaling is involved in Cd-induced osteoporosis.

5. Conclusion

In conclusion, Cd exposure causes osteoporosis through inhibiting osteoblast and osteoclast differentiation, and by promoting osteoclast and osteoblast apoptosis. Cd-induced osteoporosis is closely related to P2X7/PI3K/AKT signaling and the RANKL/OPG system. These results demonstrated that Cd-induced osteoporosis is associated with P2X7/PI3K/AKT signaling-mediated osteoblast and osteoclast differentiation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.141638.

CRediT authorship contribution statement

These authors contributed equally to this work.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgment

The manuscript was edited for English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English-speaking editors at ELIXIGEN.

This work was supported by the National Natural Science Foundation of China (Nos. 31872533, 31702204, 31502128 and 31672620), the National Key Research and Development Program of China (No. 2016YFD0501208) and the project of the Priority Academic Program Development of Jiangsu Higher Education Institutions (PADP).

References

Agrawal, A., Buckley, K.A., Bowers, K., Furber, M., Gallagher, J.A., Gartland, A., 2010. The effects of P2X7 receptor antagonists on the formation and function of human osteoclasts in vitro. Purinergic Signalling 6, 307–315.

Arbon, K.S., Christensen, C.M., Harvey, W.A., Heggland, S.J., 2012. Cadmium exposure activates the ERK signaling pathway leading to altered osteoblast gene expression and apoptotic death in Saos-2 cells. Food and Chemical Toxicology 50, 198–205.

Chen, X., Zhu, G., Jin, T., Zhou, Z., Gu, S., Qiu, J., et al., 2012. Cadmium stimulates the osteoclastic differentiation of RAW264.7 cells in presence of osteoblasts. Biol. Trace Elem. Res. 146, 349–353.

Chen, X., Wang, G., Li, X., Gan, C., Zhu, G., Jin, T., et al., 2013. Environmental level of cadmium exposure stimulates osteoclasts formation in male rats. Food and Chemical Toxicology 60, 530–535.

Chen, X., Ren, S., Zhu, G., Wang, Z., Wen, X., 2017. Emodin suppresses cadmium-induced osteoporosis by inhibiting osteoclast formation. Environ. Toxicol. Pharmacol. 54, 162–168.

Chen, K.L., Wang, H.L., Jiang, L.Z., Qian, Y., Yang, C.X., Chang, W.W., et al., 2020a. Heat stress induces apoptosis through disruption of dynamic mitochondrial networks in dairy cow mammary epithelial cells. In Vitro Cellular & Developmental Biology 56, 322–331.

Chen, S.J., Bao, L., Keefer, K., Shanmughapriya, S., Chen, L., Lee, J., et al., 2020b. Transient receptor potential ion channel TRPM2 promotes AML proliferation and survival through modulation of mitochondrial function, ROS, and autophagy. Cell Death Dis. 11, 247.

Dong, Y., Chen, Y., Zhang, L., Tian, Z., Dong, S., 2020. P2X7 receptor acts as an efficient drug target in regulating bone metabolism system. Biomed. Pharmacother. 125, 110010.

Duranova, H., Martiniakova, M., Omelka, R., Grosskopf, B., Bobonova, I., Toman, R., 2014. Changes in compact bone microstructure of rats subchronically exposed to cadmium. Acta Vet. Scand. 56, 64.

Fan, R., Hu, P.C., Wang, Y., Lin, H.Y., Su, K., Feng, X.S., et al., 2018. Betulinic acid protects mice from cadmium chloride-induced toxicity by inhibiting cadmium-induced apoptosis in kidney and liver. Toxicol. Lett. 299, 56–66.

Garcia-Guzman, M., Soto, F., Laube, B., Stühmer, W., 1996. Molecular cloning and functional expression of a novel rat heart P2X purinoceptor. FEBS Lett. 388, 123–127.

Garré, J.M., Silva, H.M., Lafaille, J.J., Yang, G., 2020. P2X7 receptor inhibition ameliorates dendritic spine pathology and social behavioral deficits in Rett syndrome mice. Nat. Commun. 11, 1784.

Gartland, A., Buckley, K.A., Bowler, W.B., Gallagher, J.A., 2003a. Blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts in vitro. Calcif. Tissue Int. 73, 361–369.

Gartland, A., Buckley, K.A., Hipskind, R.A., Perry, M.J., Tobias, J.H., Buell, G., et al., 2003b. Multinucleated osteoclast formation in vivo and in vitro by P2X7 receptor-deficient mice. Crit. Rev. Eukaryot. Gene Expr. 13, 243–253.

Genetos, D.C., Karin, N.J., Geist, D.J., Donahue, H.J., Duncan, R.L., 2011. Purinergic signaling is required for fluid shear stress-induced NF-κB translocation in osteoblasts. Exp. Cell Res. 317, 737–744.

Grol, M.W., Zelner, I., Dixon, S.J., 2012. $P2X_7$-mediated calcium influx triggers a sustained, PI3K-dependent increase in metabolic acid production by osteoblast-like cells. Am. J. Physiol. Endocrinol. Metab. 302, E561–E575.
Gu, B.J., Zhang, W., Worthington, R.A., Sluyter, R., Dao-Ung, P., Petrou, S., et al., 2001. A Glu-496 to Ala polymorphism leads to loss of function of the human P2X7 receptor. J. Biol. Chem. 276, 11135–11142.
Hartwig, A., 2013. Cadmium and cancer. Metal Ions in Life Sciences 11, 491–507.
He, S., Zhuo, L., Cao, Y., Liu, G., Zhao, H., Song, R., et al., 2020. Effect of cadmium on osteoclast differentiation during bone injury in female mice. Environ. Toxicol. 35, 487–494.
Heitzer, E., Auinger, L., Speicher, M.R., 2020. Cell-free DNA and apoptosis: how dead cells inform about the living. Trends Mol. Med. 26, 519–528.
Hu, K.H., Li, W.X., Sun, M.Y., Zhang, S.B., Fan, C.X., Wu, Q., et al., 2015. Cadmium induced apoptosis in MG63 cells by increasing ROS, activation of p38 MAPK and inhibition of ERK 1/2 pathways. Cellular Physiology and Biochemistr 36, 642–654.
Husted, L.B., Harsløf, T., Stenkjær, L., Carstens, M., Jørgensen, N.R., Langdahl, B.L., 2013. Functional polymorphisms in the P2X7 receptor gene are associated with osteoporosis. Osteoporos. Int. 24, 949–959.
Iwami, K., Moriyama, T., 1993. Comparative effect of cadmium on osteoblastic cells and osteoclastic cells. Arch. Toxicol. 67, 352–357.
Kar, I., Mukhopadhayay, S.K., Patra, A.K., Pradhan, S., 2018. Bioaccumulation of selected heavy metals and histopathological and hematobiochemical alterations in backyard chickens reared in an industrial area, India. Environ. Sci. Pollut. Res. Int. 25, 3905–3912.
Knani, L., Bartolini, D., Kechiche, S., Tortoioli, C., Murdolo, G., Moretti, M., et al., 2019. Melatonin prevents cadmium-induced bone damage: first evidence on an improved osteogenic/adipogenic differentiation balance of mesenchymal stem cells as underlying mechanism. J. Pineal Res. 67, e12597.
Korcok, J., Raimundo, L.N., Ke, H.Z., Sims, S.M., Dixon, S.J., 2004. Extracellular nucleotides act through P2X7 receptors to activate NF-kappaB in osteoclasts. J. Bone Miner. Res. 19, 642–651.
Liu, D., Genetos, D.C., Shao, Y., Geist, D.J., Li, J., Ke, H.Z., et al., 2008. Activation of extracellular-signal regulated kinase (ERK1/2) by fluid shear is Ca(2+)- and ATP-dependent in MC3T3-E1 osteoblasts. Bone 42, 644–652.
Liu, W., Dai, N., Wang, Y., Xu, C., Zhao, H., Xia, P., et al., 2016. Role of autophagy in cadmium-induced apoptosis of primary rat osteoblasts. Sci. Rep. 6, 20404.
Lu, J., Kuang, Z., Chen, T., Ye, C., Hou, W., Tang, L., et al., 2020. Isoalantolactone inhibits RANKL-induced osteoclast formation via multiple signaling pathways. Int. Immunopharmacol. 84, 106550.
Luo, H.F., Zhang, J.Y., Jia, W.J., Ji, F.M., Yan, Q., Xu, Q., et al., 2017. Analyzing the role of soil and rice cadmium pollution on human renal dysfunction by correlation and path analysis. Environ. Sci. Pollut. Res. Int. 24, 2047–2054.
Lv, Y.J., Wei, Q.Z., Zhang, Y.C., Huang, R., Li, B.S., Tan, J.B., et al., 2019. Low-dose cadmium exposure acts on rat mesenchymal stem cells via RANKL/OPG and downregulate osteogenic differentiation genes. Environmental Pollution (Barking, Essex: 1987) 249, 620–628.
Ma, Y., Zhao, H., Chile, C., Wang, C., Zheng, J., Song, R., et al., 2019. The effect of P2X7R-mediated Ca signaling in OPG-induced osteoclasts adhesive structure damage. Exp. Cell Res. 383, 123–134.
Miyahara, T., Takata, M., Mori-Uchi, S., Miyata, M., Nagai, M., Sugure, A., et al., 1992. Stimulative effects of cadmium on bone resorption in neonatal parietal bone resorption. Toxicology 73, 93–99.
Nazima, B., Manoharan, V., Miltonprabu, S., 2015. Grape seed proanthocyanidins ameliorates cadmium-induced renal injury and oxidative stress in experimental rats through the up-regulation of nuclear related factor 2 and antioxidant responsive elements. Biochemistry and Cell Biology 93, 210–226.
Nishijo, M., Nakagawa, H., Suwazono, Y., Nogawa, K., Kido, T., 2017a. Causes of death in patients with Itai-itai disease suffering from severe chronic cadmium poisoning: a nested case-control analysis of a follow-up study in Japan. BMJ Open 7, 234–245.
Nishijo, M., Nakagawa, H., Suwazono, Y., Nogawa, K., Kido, T., 2017b. Causes of death in patients with Itai-itai disease suffering from severe chronic cadmium poisoning: a nested case-control analysis of a follow-up study in Japan. BMJ Open 7, e015694.
Noronha-Matos, J.B., Coimbra, J., Sá-e-Sousa, A., Rocha, R., Marinhas, J., R, F., et al., 2014. P2X7-induced zeiosis promotes osteogenic differentiation and mineralization of postmenopausal bone marrow-derived mesenchymal stem cells. FASEB Journal 28, 5208–5222.
Oboh, G., Adebayo, A.A., Ademosun, A.O., Olowokere, O.G., 2020. Rutin restores neurobehavioral deficits via alterations in cadmium bioavailability in the brain of rats exposed to cadmium. Neurotoxicology 77, 12–19.
Orisakwe, O.E., Oladipo, O.O., Ajaezi, G.C., Udowelle, N.A., 2017. Horizontal and vertical distribution of heavy metals in farm produce and livestock around lead-contaminated goldmine in Dareta and Abare, Zamfara State, Northern Nigeria. J. Environ. Public Health 2017, 3506949.
Orriss, I.R., Key, M.L., Brandao-Burch, A., Patel, J.J., Burnstock, G., Arnett, T.R., 2012. The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: the role of p2x receptors. Bone 51, 389–400.
Panupinthu, N., Rogers, J.T., Zhao, L., Solano-Flores, L.P., Possmayer, F., Sims, S.M., et al., 2008. P2X7 receptors on osteoblasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis. J. Cell Biol. 181, 859–871.
Radwan, M.A., Salama, A.K., 2006. Market basket survey for some heavy metals in Egyptian fruits and vegetables. Food Chem. Toxicol. 44, 1273–1278.
Rana, K., Verma, Y., Rana, S.V.S., 2020. Possible mechanisms of liver injury induced by cadmium sulfide nanoparticles in rat. Biol. Trace Elem. Res. 10, 10–21.
Rodríguez, J., Mandalunis, P.M., 2016. Effect of cadmium on bone tissue in growing animals. Experimental and Toxicologic Pathology 68, 391–397.
Soysa, N.S., Alles, N., 2016. Osteoclast function and bone-resorbing activity: an overview. Biochem. Biophys. Res. Commun. 476, 115–120.
Sughis, M., Penders, J., Haufroid, V., Nemery, B., Nawrot, T.S., 2011. Bone resorption and environmental exposure to cadmium in children: a cross-sectional study. Environmental Health 10, 104.
Sun, D., Junger, W.G., Yuan, C., Zhang, W., Bao, Y., Qin, D., et al., 2013. Shockwaves induce osteogenic differentiation of human mesenchymal stem cells through ATP release and activation of P2X7 receptors. Stem Cells (Dayton, Ohio) 31, 1170–1180.
Sun, P., Wang, M., Yin, G.Y., 2020. Endogenous parathyroid hormone (PTH) signals through osteoblasts via RANKL during fracture healing to affect osteoclasts. Biochem. Biophys. Res. Commun. 525, 850–856.
Volonté, C., Apolloni, S., Parisi, C., Amadio, S., 2016. Purinergic contribution to amyotrophic lateral sclerosis. Neuropharmacology 104, 180–193.
Wang, Y., Fu, Y.X., Gu, J.H., Yuan, Y., Liu, X.Z., Bian, J.C., et al., 2014. Cadmium induces the differentiation of duck embryonic bone marrow cells into osteoclasts in vitro. Vet. J. 200, 181–185.
Wang, D., Sun, H., Wu, Y., Zhou, Z., Ding, Z., Chen, X., et al., 2016. Tubular and glomerular kidney effects in the Chinese general population with low environmental cadmium exposure. Chemosphere 147, 3–8.
Xiong, L., Jung, J.U., Guo, H.H., Pan, J.X., Sun, X.D., Mei, L., et al., 2017. Osteoblastic Lrp4 promotes osteoclastogenesis by regulating ATP release and adenosine-A(2A)R signaling. J. Cell Biol. 216, 761–778.
Xu, S., Bao, K., Shu, B., 1997. Influence of cadmium on cartilage and bone formation induced by bone morphogenetic protein. Chinese Journal of Preventive Medicine 31, 292–294.
Zhang, D., Gao, J., Zhang, K., Liu, X., Li, J., 2012. Effects of chronic cadmium poisoning on Zn, Cu, Fe, Ca, and metallothionein in liver and kidney of rats. Biol. Trace Elem. Res. 149, 57–63.
Zhang, Y., Li, W., Liu, C., Yan, J., Yuan, X., Wang, W., et al., 2019. Electromagnetic field treatment increases purinergic receptor P2X7 expression and activates its downstream Akt/GSK3β/β-catenin axis in mesenchymal stem cells under osteogenic induction. Stem Cell Res Ther 10, 407.