

The Effects of 2-Aminoethoxydiphenyl Borate (2-APB) and Dantrolene on Rat Bones Treated With NaAsO₂

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The effects of 2-aminoethoxydiphenyl borate (2-APB) and dantrolene on rat bones treated with NaAsO₂

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[Abstract]

Objective To observe the effects of arsenic and the protective effects of 2-aminoethoxydiphenyl borate (2-APB) and dantrolene on the trabeculae of Sprague Dawley (SD) rats. **Methods** Thirty-six SD rats were randomly divided into a control group and an arsenic poisoning group. After 12 weeks of arsenic exposure, six rats in the arsenic poisoning group were randomly selected for sacrifice. The remaining rats were randomly assigned to four groups (n=6 per group): natural recovery after arsenic exposure, dantrolene intervention after arsenic exposure, 2-APB intervention after arsenic exposure, and 2-APB + dantrolene intervention after arsenic exposure. After 21 days of treatment by oral gavage every other day, the bilateral femurs and tibiae of the rats were scanned using micro-computed tomography (micro-CT). Bone marrow stromal cells (BMSCs) were isolated and cultured, and Ca^{2+} concentration and alkaline phosphatase (ALP) activity of BMSCs was assessed. **Results** Compared with the control group, bone mineralization density (BMD), bone volume (BV)-to-total volume (TV) ratio (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and degree of anisotropy (DA) decreased, while trabecular separation (Tb.Sp), trabecular pattern factor (TBpf), and structural model index (SMI) increased in the arsenic poisoning group ($P<0.05$); additionally, Ca^{2+} concentration increased and ALP activity decreased significantly in the arsenic poisoning group ($P<0.05$). Compared with the natural recovery group, after arsenic exposure, the indices of micro-CT recovered to some extent, the Ca^{2+} concentration of BMSCs decreased significantly, and the ALP

activity of BMSCs increased significantly after intervention with 2-APB and dantrolene ($P<0.05$). **Conclusion** Arsenic can lead to significant changes in the structure of trabeculae and osteoporosis in rats, and these changes can be improved by intervention with 2-APB and dantrolene.

[Keywords] NaAsO₂ 2-Aminoethoxydiphenyl borate (2-APB) Dantrolene osteoporosis Micro-computed tomography (Micro-CT)

Introduction

Arsenic poisoning is a major public health problem and the main cause of poisoning different systems of the human body^[1]. Previous studies have reported that arsenic exposure can lead to osteopenia and increase the risk of osteoporosis in the population^[2]. Ca²⁺ channel blockers 2-APB and dantrolene play an important role in maintaining intracellular Ca²⁺ homeostasis^[3-5]. Therefore, the major objective of this study was to observe the effect of arsenic on the trabeculae of Sprague Dawley (SD) rats and test the protective effects of 2-APB and dantrolene. In this study, an arsenic exposure model and a bone marrow stromal cell (BMSC) anti-arsenic cell model was established. This study provides a scientific basis for research on bone injury caused by arsenic, as well as potential recovery mechanisms.

1 Materials and Methods

1.1 Experimental Animals and Grouping

Thirty-six male SPF grade Sprague-Dawley (SD) rats (200 ± 20 g; 12 weeks old) were obtained from the Animal Experimental Center of the First Affiliated Hospital of Xinjiang Medical University. The rats were housed under the following optimum environmental conditions: temperature, 21 ± 2 °C; humidity, 40-60%. The rats were randomly divided into a control group ($n = 6$) and an arsenic poisoning group ($n = 30$). NaAsO_2 was prepared with distilled water to a 10 mg/kg solution for the arsenic poisoning group, while the control group received distilled water. Rats in both groups were fed with common feedstuffs. After 12 weeks of arsenic exposure, six randomly selected rats in the arsenic poisoning group and 6 rats in the control group were sacrificed. In the arsenic poisoning, the remaining rats were randomly assigned to four groups ($n = 6$ per group), as follows: natural recovery after arsenic exposure, 20 $\mu\text{mol/L}$ dantrolene intervention after arsenic exposure, 20 $\mu\text{mol/L}$ 2-APB intervention after arsenic exposure, 20 $\mu\text{mol/L}$ 2-APB + 20 $\mu\text{mol/L}$ dantrolene combined intervention after arsenic exposure. Dantrolene and 2-APB were diluted at a dose of 1 mL/100 g. The drug was administered by oral gavage every other day for 21 days. All methods were carried out in accordance with the guidelines and regulations of experimental animals approved by the Medical Ethics Committee of the First Affiliated Hospital of

Medical College, Shihezi University. The study was carried out in compliance with the ARRIVE guidelines for the reporting of animal experiments.

1.2 Instruments and reagents

NaAsO₂, 2-aminoethoxydiphenyl borate (2-APB), and dantrolene were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sky scan Micro 1176 Micro CT and Micro View image processing software (related software of 2D/3D graphic analysis, bone morphology and simulation visualization) were purchased from Brooke (Bruker) Scientific Instruments.

1.3 Sampling and Specimen Preparation

The rats in each group were fasted for 24 h before sacrifice with free access to drinking water. The fur of rats was soaked with 75% alcohol for disinfection. The skin of the lower limbs was cut, the bilateral femurs and tibias were removed using tweezers and tissue scissors, and the surrounding fat and muscle were removed. The bone samples were then placed in 4% paraformaldehyde solution for fixation.

1.4 Micro-computed tomography (CT) Scanning

The specimens were placed in the scanning area of the micro-CT in order and were

fixed using tape. Scanning was performed through 360 ° rotation with a 0.7° rotation angle increment, 80 kV power, 500 µA current, 9 µm resolution, 10 mm scanning length, and 32 min scanning time. The scanning direction was along the long axis of the specimens, and the center of the femur and tibia was the tibial plateau. Finally, more than 500 consecutive micro-CT images of different sections were obtained from the same specimen. The trabecular area of the femurs and the medial tibias were manually delimited as the regions of interest (ROIs), and this area was three-dimensionally reconstructed. The reconstructed image was assessed on a CT data viewer, CTan, and CTvol to calculate three-dimensional stereometric parameters and bone mineral density parameters, and these parameters were analyzed quantitatively.

1.5 Isolation and Culture of BMSCs

The rats in this study were sacrificed and the entire body was immersed in 75% alcohol solution for 30 min. The bilateral femurs and tibias of the rats were removed under aseptic conditions, and both ends of the femurs and tibias were removed. L-DMEM (5 mL) was used to wash the bone marrow cavity. Then, the bone marrow was washed with L-DMEM in a petri dish, moved into a 15-mL aseptic centrifuge tube, and centrifuged at 1000 rpm for 5 min. The supernatant and fat layer were discarded, and the precipitate was mixed with L-DMEM. After 24 h, the medium was changed, non-adherent cells were discarded, cells were counted, and the density was adjusted. The

cells were inoculated in a petri dish at a density of 1×10^9 cells/L and incubated at 37 °C under 5% CO₂. The medium was changed every 2 days. When cells reached 80-90% confluence (passage 0), they were passaged using 0.25% trypsin. Cells from the third passage were used in subsequent experiments.

1.6 Inducing Osteoblasts

Cells from the third passage were inoculated at a density of 1×10^5 cells/mL in a 6-wells culture plate with cover slides. When cells reached 80-90% confluence, the medium was changed to osteoblast differentiation medium containing 0.1 μmol/L dexamethasone, 10 mmol/L β-glycerophosphate sodium, 50 μmol/L vitamin C, 100 U/mL double antibody, and 100 mL/L fetal bovine serum.

1.7 Determination of Ca²⁺ Concentration using Flow Cytometry

2 mM of Ca²⁺ fluorescent probes Fluo-4 and AM were prepared in advance. The concentration of Fluo-4 and AM was diluted to 5 μM, which was the optimum concentration for the determination of Ca²⁺ in BMSCs. Fluo-4 and AM were added to the cells in 6-well plates and incubated at 37 °C for 20 min. After incubation, Hank's Balanced Salt Solution (HBSS) containing 1% fetal bovine serum was added to the cells, which were then incubated for 40 min. Then, the cells were washed with HEPES buffer, the control group and arsenic poisoning group were removed, and trypsin

without EDTA was added. During this period, the trypsin was continuously mixed, and fetal bovine serum was added to stop digestion. After centrifugation, the precipitate was collected and washed with PBS twice. Then, HEPES buffer was used to resuscitate the cells, to prepare a 1×10^5 cells/mL cell suspension. The cell suspension was incubated at 37 °C for 10 min, and the Ca^{2+} concentration of the cells was detected using flow cytometry. Detection conditions were as follows: excitation wavelength of 488 nm, emission wavelength of 516 nm, and FITC channel.

1.8 Determination of alkaline phosphatase (ALP) Activity using Visible Light Colorimetry

Cells from the third passage were inoculated at a density of 1×10^5 cells/mL in a 24-well culture plate, and 1 mL of the cell suspension was inoculated into each well. Osteoblast differentiation induction medium was added to each well, and the same sample size was used for the control. On day 14 of differentiation, three wells of the experimental group and three wells of the control group were digested with 2.5 g/L trypsin and were washed twice with PBS. The cells of each well were treated with 1 mL PBS and 200 W of the ultrasonic crusher three times, each time for 3 s at intervals of 2 min. After centrifugation, the supernatant was removed to determine the ALP activity (the ALP activity was determined by measuring the absorbance increase rate at 520nm in an alkaline environment using a microplate reader).

1.9 Statistical Methods

The experiments presented were repeated three times. Statistical analyses were performed using SPSS 22.0. All data are presented as the mean \pm standard deviation. The means of two groups were compared using the LSD-t test, and the means of multiple groups were compared using one-way analysis of variance (ANOVA). $\alpha = 0.05$ was considered the confidence level, and $P < 0.05$ was considered statistically significant.

2 Results

2.1 Micro-CT Scanning

2.1.1 Microstructure of trabeculae

2.1.1.1 Two-dimensional imaging

In the control group, the structure of the trabeculae was clear. There were many trabeculae, the distribution of which was uniform and the structure of which was not broken; moreover, the distance between the trabeculae was average and continuous. In

the arsenic poisoning group, the structure of trabeculae was not clear, rather it was sparse or even absent. The number of trabeculae was significantly lower and had a disordered arrangement. The distribution of trabeculae was uneven, the structure of some trabeculae was broken, and microfracture was observed. The distance between the trabeculae was larger and wider, and their continuity was incomplete. In addition, there was a cystic change area in the sample, showing a low-density shadow. After intervention with 2-APB and dantrolene, the structure of the trabeculae was significantly improved. Although the distribution of trabeculae was still uneven, the structure of the trabeculae was clearly visible, the number of trabeculae increased, the lamellar structure was obvious, and the continuity of trabeculae improved (Fig. 1).

2.1.1.2 Three-dimensional imaging

In the control group, there were many trabeculae with clear structures. In the arsenic poisoning group, the structure of the trabeculae was broken, the spacing was sparse, and the cavity was more significant. After intervention with 2-APB and dantrolene, the structure of the trabeculae effectively improved, the distance between the trabeculae was effectively restored, the structure was clearly visible, and continuity was significant (Fig. 2).

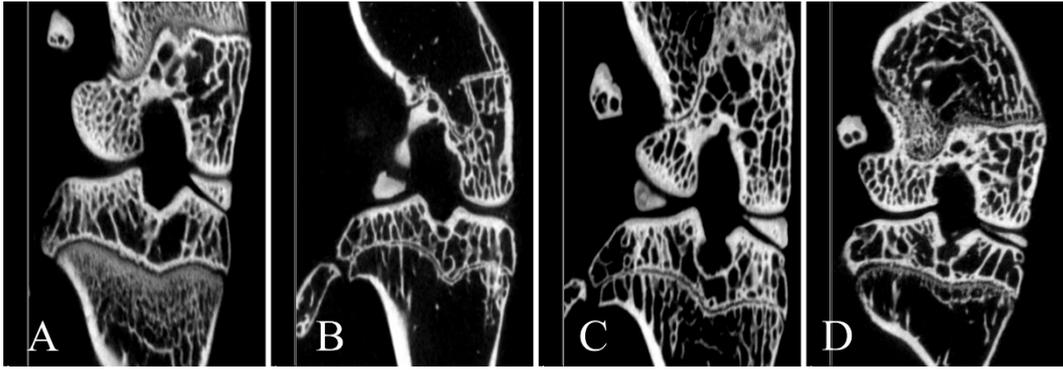


Fig. 1 Two-dimensional microstructure of trabeculae

A: Control group; B: Arsenic poisoning group; C: 2-APB intervention after arsenic exposure; D: 2-APB + Dantrolene combined intervention after arsenic exposure.

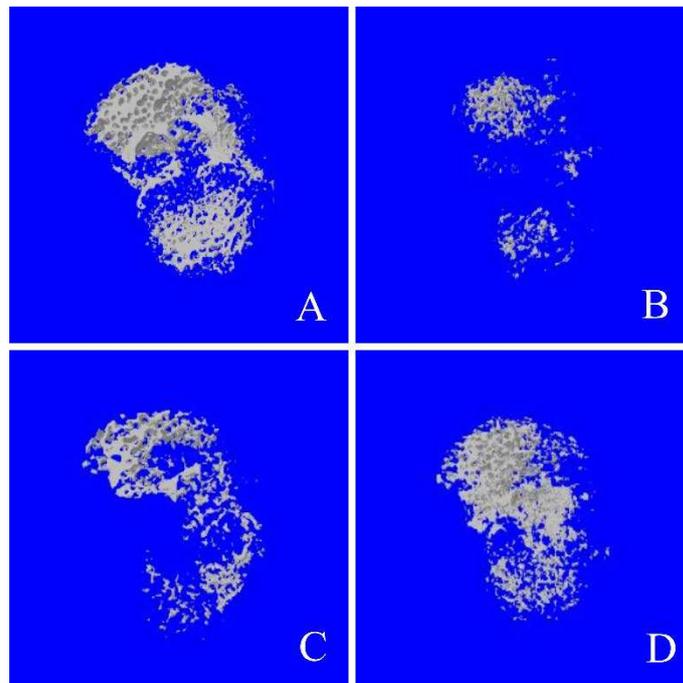


Fig. 2 Three-dimensional microstructure of trabeculae

A: Control group; B: Arsenic poisoning group; C: 2-APB intervention after arsenic exposure; D: 2-APB + Dantrolene combined intervention after arsenic exposure.

2.1.2 Parameters

Compared with the control group, bone mineralization density (BMD), bone volume (BV)-to-total volume (TV) ratio (BV/TV), trabecular number Tb.N (1/mm), trabecular thickness Tb.Th (μm), and degree of anisotropy (DA) decreased, while trabecular separation Tb.Sp (mm), trabecular pattern factor TBpf (1/mm), and structural model index (SMI) increased in the arsenic poisoning group. Importantly, intervention with 2-APB and dantrolene recovered the indices of micro-CT to some extent ($P < 0.05$) (Fig. 3,4).

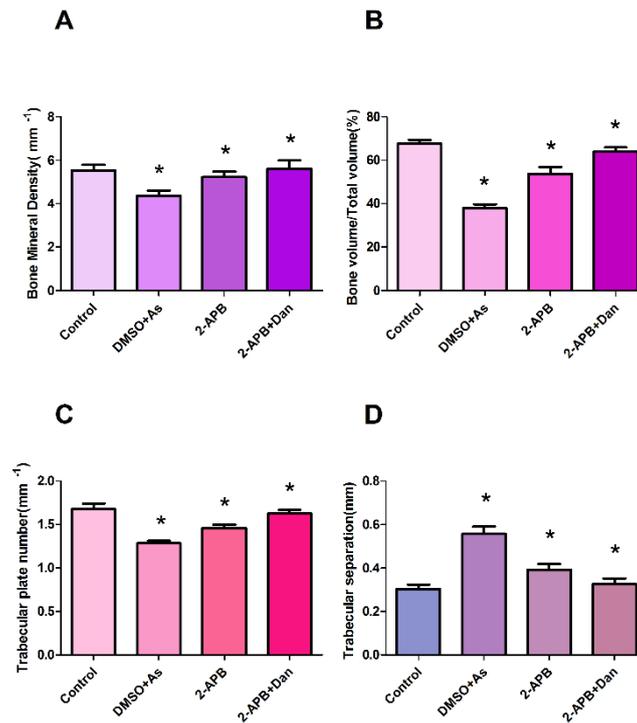


Fig. 3 Bone metrological analysis

A: BMD; B: BV/TV; C: Tb.N; D: Tb.Sp

* Compared with the control group, $P < 0.05$.

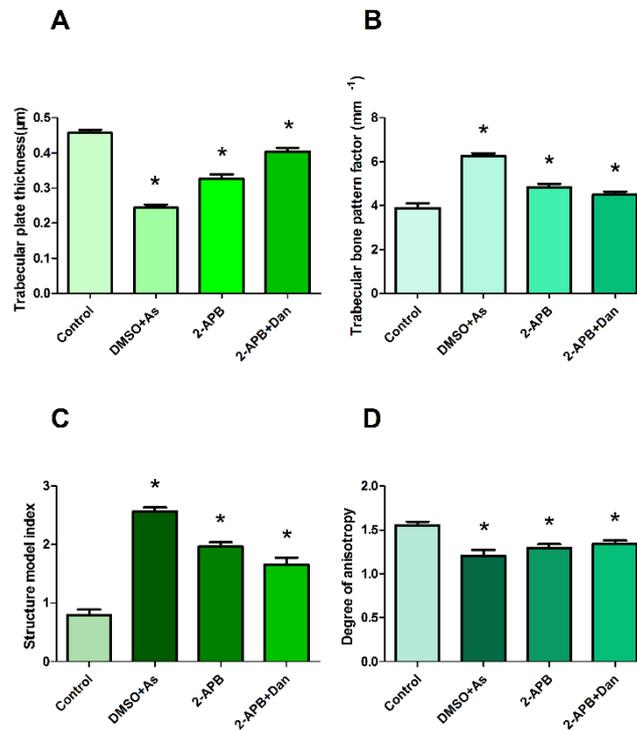


Fig. 4 Bone metrological analysis

A: Tb.Th; B: TBpf; C: SMI; D: DA

* Compared with the control group, $P < 0.05$.

2.2 Primary culture of BMSCs

After BMSC culture for 24 h, most of the cells adhered to the wall and began to divide and proliferate, showing colony growth. With the extension of culture time, the colonies increased, expanded, fused with each other, and covered the bottom of the culture flask by the second day. The passaged cells adhered quickly, distributed

uniformly in fusiform, and proliferated rapidly (Fig. 5). Cells from the third passage were selected for this experiment. Bone marrow mesenchymal stem cells were stained with alizarin red during the differentiation of rat bone marrow mesenchymal stem cells into osteoblasts (Fig. 6).

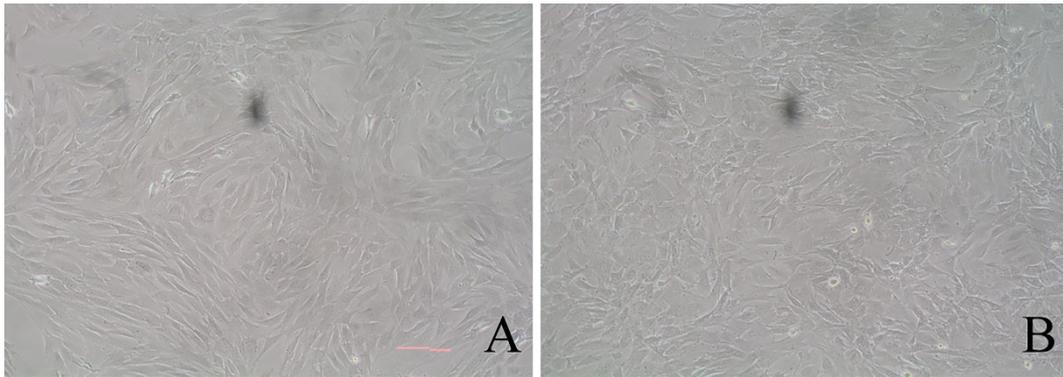


Fig. 5 Primary culture of BMSCs

A.B Third generation BMSCs ($\times 100$)

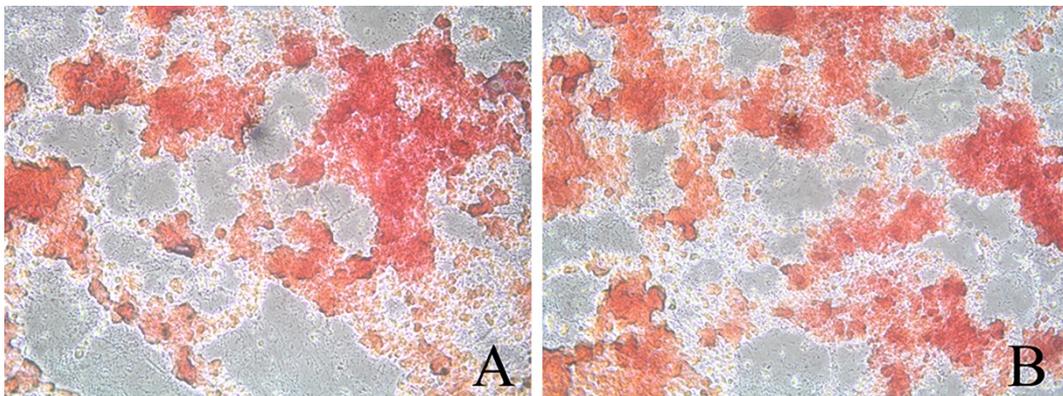
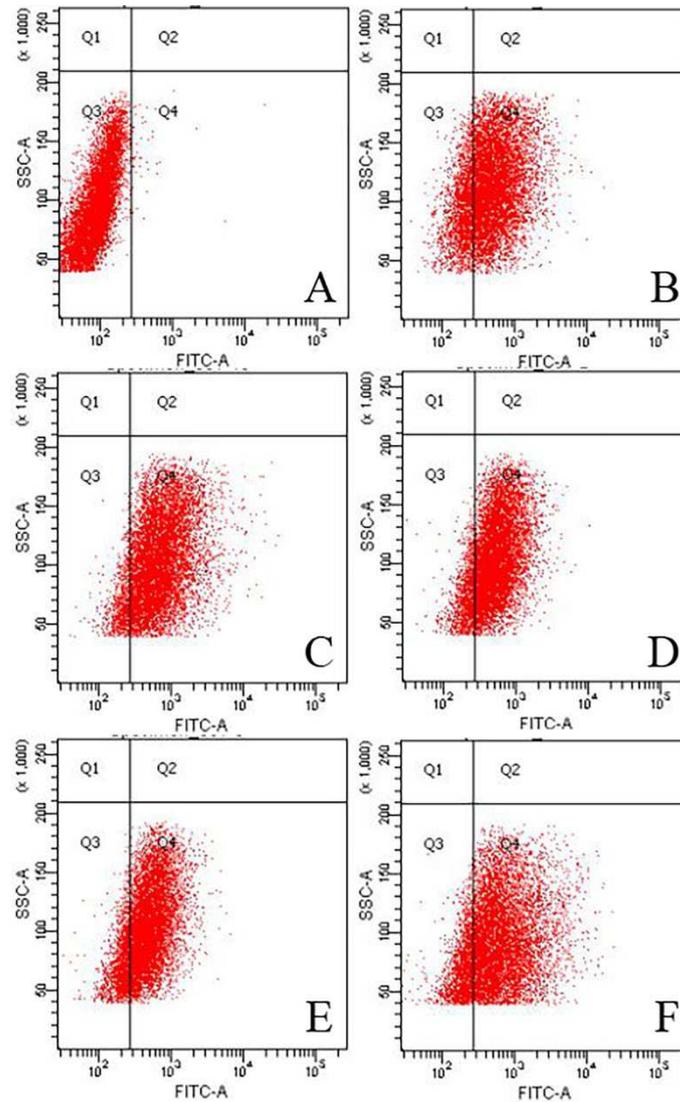


Fig. 6 Osteogenic differentiation of BMSCs

A.B Osteoblasts differentiated from third generation BMSCs ($\times 100$)

2.3 Concentration of Ca²⁺ in BMSCs

Compared with the control group, the concentration of Ca²⁺ in BMSCs increased in the arsenic poisoning group. The Ca²⁺ concentration decreased after intervention with 2-APB and dantrolene alone. Furthermore, after combined intervention of 2-APB and dantrolene, the concentration of Ca²⁺ in BMSCs decreased more significantly (Fig 7).



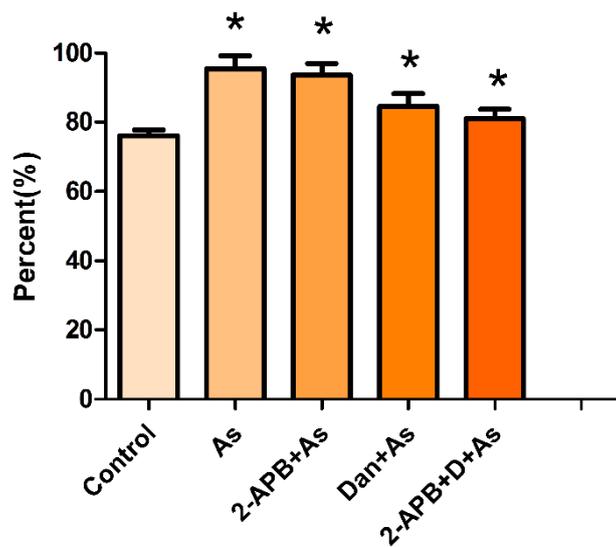


Fig. 7 Ca^{2+} concentration of BMSCs

A: blank group; B: control group; C: arsenic poisoning group; D: dantrolene intervention after arsenic exposure; E: 2-APB intervention after arsenic exposure; F: 2-APB + dantrolene combined intervention after arsenic exposure.

2.4 ALP activity of BMSCs

Compared with the control group, the ALP activity of BMSCs decreased significantly in the arsenic poisoning group, and the ALP activity increased after intervention with 2-APB and dantrolene (Fig. 8).

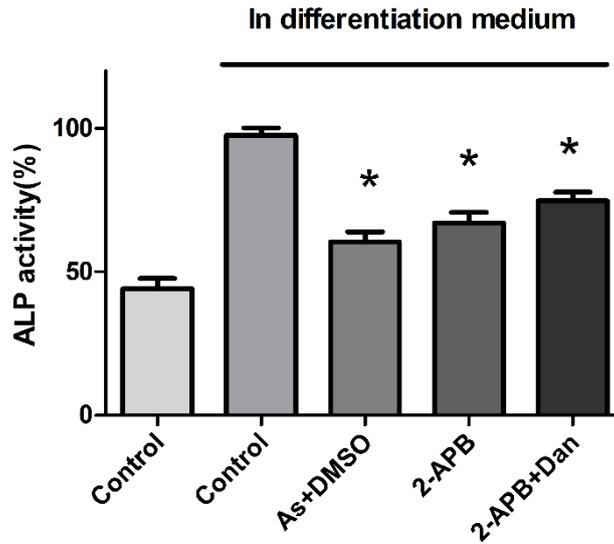


Fig. 8 ALP activity of BMSCs

* Compared with the control group, $P < 0.05$.

3 Discussion

Arsenic can have toxic effects on different systems of the human body. Arsenic may replace the position of phosphate in hydroxyapatite crystals, thereby affecting bone mineralization and increasing the risk of osteoporosis^[6, 7]. However, at present, the mechanism of arsenic-induced osteoporosis is not well understood, and there is a relative lack of clinical research on the treatment of arsenic-induced osteoporosis^[8]. Micro-CT, as a new imaging technology, is non-invasive and has high precision and resolution^[9, 10]. It can obtain three-dimensional information of complex structures, evaluate bone mineral density and bone structure, and provide an estimate of the

pathological state of bone^[10]. As an ideal target cell for gene therapy, BMSCs have high metabolic activity and can effectively facilitate the secretion of recombinant proteins^[11]. BMSCs play a key role in the process of bone metabolism with a strong ability to repair osteonecrosis and maintain a strong osteogenic potential^[11].

In this study, BV/TV, Tb.N, Tb.Th, and DA decreased, but Tb.Sp, TBpf, and SMI increased in the arsenic poisoning group, which is consistent with the results of Hu et al.^[12]. At the same time, third generation BMSCs had an increased Ca^{2+} concentration and decreased ALP activity after arsenic poisoning, which was consistent with the results of Cheng et al.^[13]. The possible reasons for the results are as follows: arsenic may replace the position of phosphate in hydroxyapatite crystals, thereby inhibiting the mineralization of the extracellular matrix and leading to a decrease in bone mass. Arsenic may also inhibit the proliferation of osteoblasts and promote the differentiation of osteoclasts, resulting in increased bone resorption, osteoporosis, and further changes in the structure of the trabeculae. After a large amount of arsenic accumulates in the body, it induces the endoplasmic reticulum stress response, disrupts the endoplasmic reticulum Ca^{2+} signal, and induces the Ca^{2+} ion in the bone to be released into the body, leading to an increase in bodily Ca^{2+} concentration. ALP is a specific indicator of the function and differentiation of osteoblasts. Arsenic may reduce the differentiation of osteoblasts induced by BMSCs in vitro, resulting in decreased ALP activity.

It was also found that compared with the natural recovery group, the related indexes of micro-CT were improved after intervention with 2-APB and dantrolene. We

consistently found that after intervention with 2-APB and dantrolene, the concentration of Ca^{2+} in BMSCs was significantly decreased and ALP activity was increased, which is consistent with the results of Claire et al.^[14]. The possible reasons for these results are as follows. Maintaining a Ca^{2+} balance is particularly important for bone metabolism, and two specific Ca^{2+} channels in the endoplasmic reticulum, Ry-R and IP3R, play an important role in the regulation of Ca^{2+} . Arsenic exposure may disrupt the endoplasmic reticulum Ca^{2+} signal, so the Ry-R inhibitor dantrolene can dissociate muscle excitation-contraction coupling by inhibiting muscle Ca^{2+} release. 2-APB, an inhibitor of IP3R, can affect Ca^{2+} influx controlled by the Ca^{2+} pool, by regulating the activity of Ca^{2+} release-activated Ca^{2+} channels. Either alone or in combination, these two drugs can improve the degree of arsenic-induced osteoporosis in rats and induce a protective effect on bone trabeculae in rats.

In conclusion, arsenic induced significant changes in the structure of trabeculae and caused osteoporosis in rats. The structure of the trabeculae could be improved by intervention with 2-APB and dantrolene. Based on the above results, we speculate that the effect of 2-APB and dantrolene on the bone is to prevent the release of Ca^{2+} and restore ALP activity, thereby treating arsenic toxicity. This experiment provides new insights and an experimental basis for the study of treatments for diseases caused by arsenic poisoning.

Abbreviations

2-APB: 2-aminoethoxydiphenyl borate; micro-CT: micro-computed tomography; BMSCs: Bone marrow stromal cells; ALP: alkaline phosphatase; BMD: bone mineralization density; BV: bone volume; TV: total volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; DA: degree of anisotropy; Tb.Sp: trabecular separation; TBpf: trabecular pattern factor; SMI: structural model index

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Authors' contributions

JF, WJQ and HLL wrote the main manuscript text, ZW and ZJZ prepared figures 1-4, LH and SSD prepared figures 5-8, GLL and YFJ reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data of this article are not publicly available because it is confidential, but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

1 The experimental protocol has passed an ethical clearance of Medical Ethics Committee of the first affiliated Hospital of Medical College, Shihezi University and the approval number is A2020-167-01.

2 All methods were carried out in accordance with the guidelines and regulations of experimental animals approved by the Medical Ethics Committee of the First Affiliated Hospital of Medical College, Shihezi University.

3 The study was carried out in compliance with the ARRIVE guidelines for the reporting of animal experiments.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author's information

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Figures

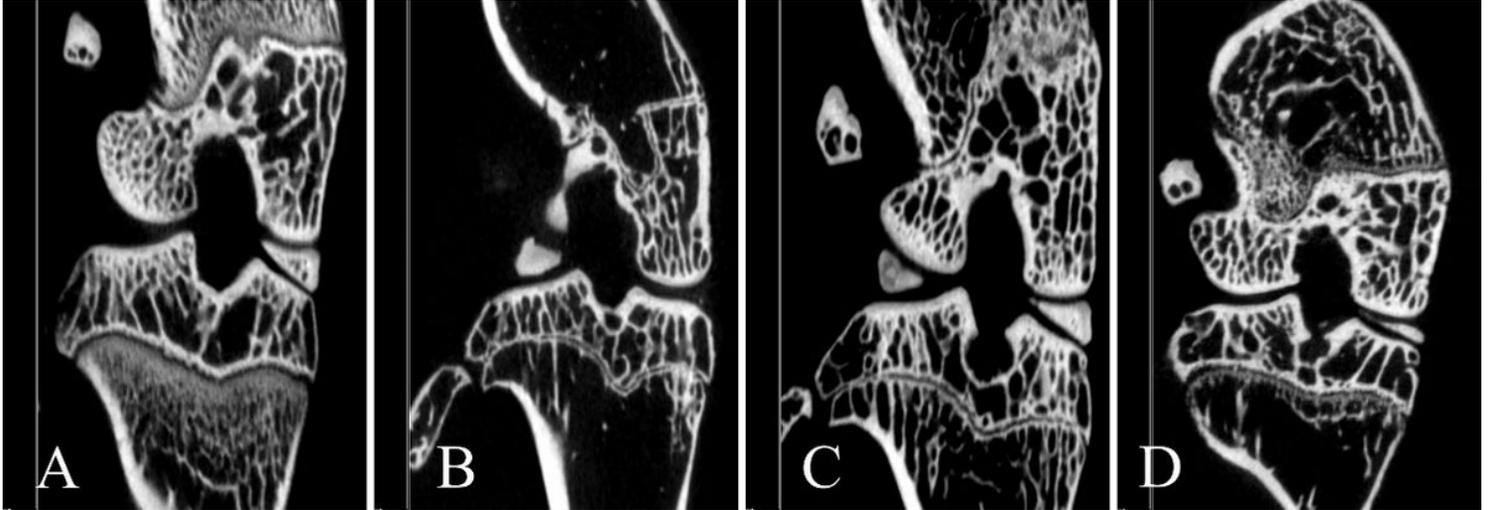


Figure 1

Two-dimensional microstructure of trabeculae A: Control group; B: Arsenic poisoning group; C: 2-APB intervention after arsenic exposure; D: 2-APB + Dantrolene combined intervention after arsenic exposure.

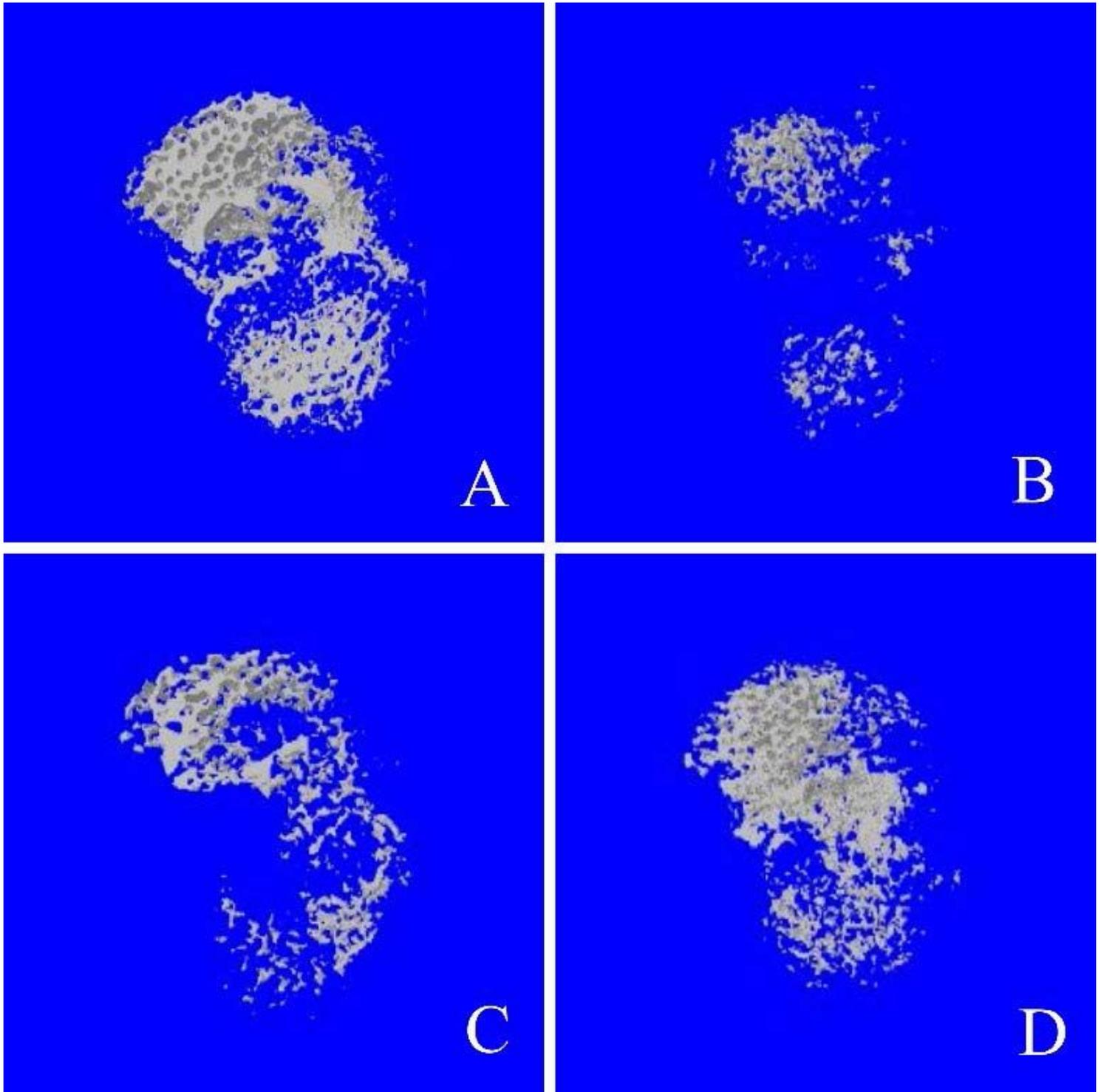


Figure 2

Three-dimensional microstructure of trabeculae A: Control group; B: Arsenic poisoning group; C: 2-APB intervention after arsenic exposure; D: 2-APB + Dantrolene combined intervention after arsenic exposure.

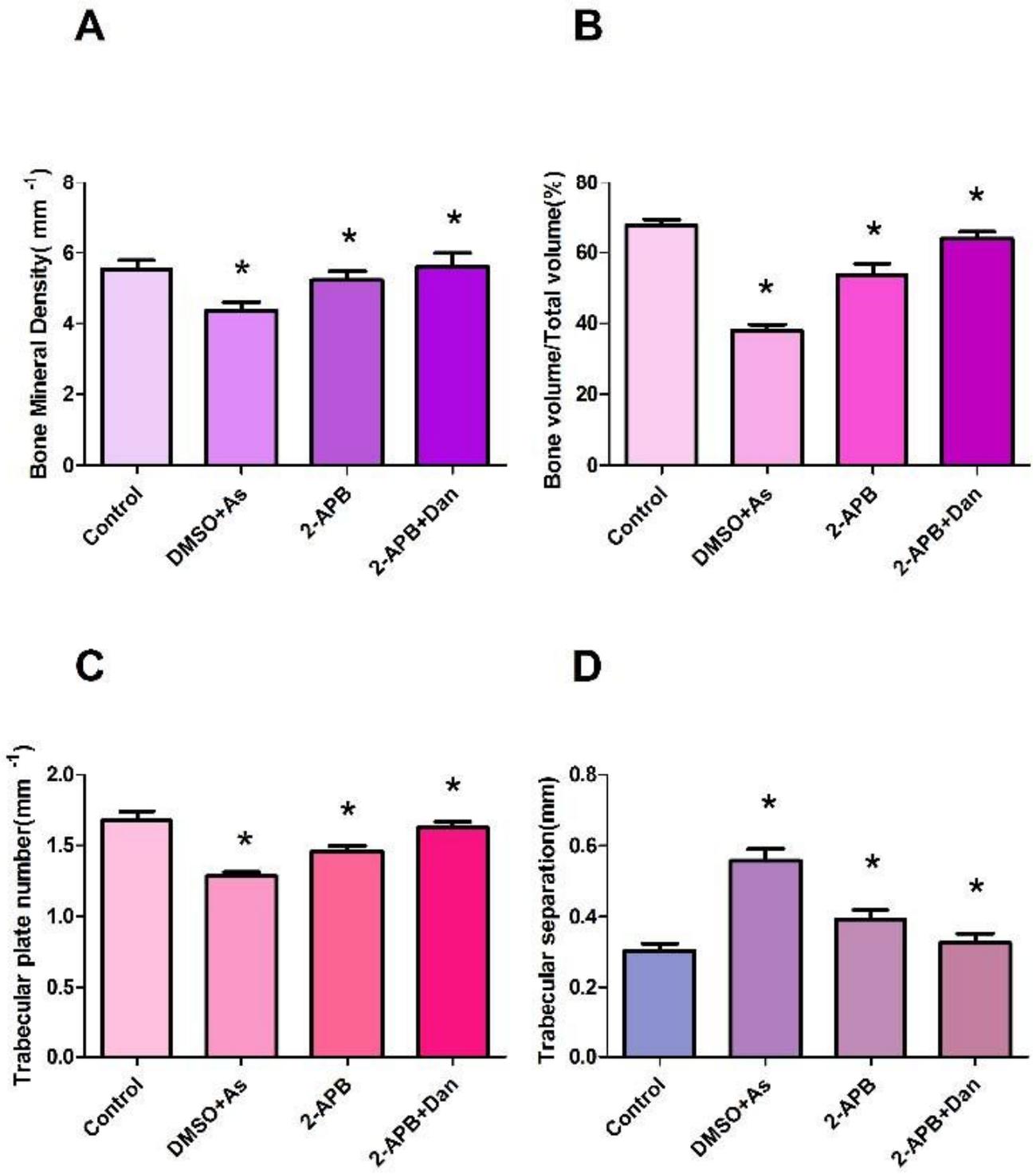


Figure 3

Bone metrological analysis A: BMD; B: BV/TV; C: Tb.N; D: Tb.Sp * Compared with the control group, $P < 0.05$.

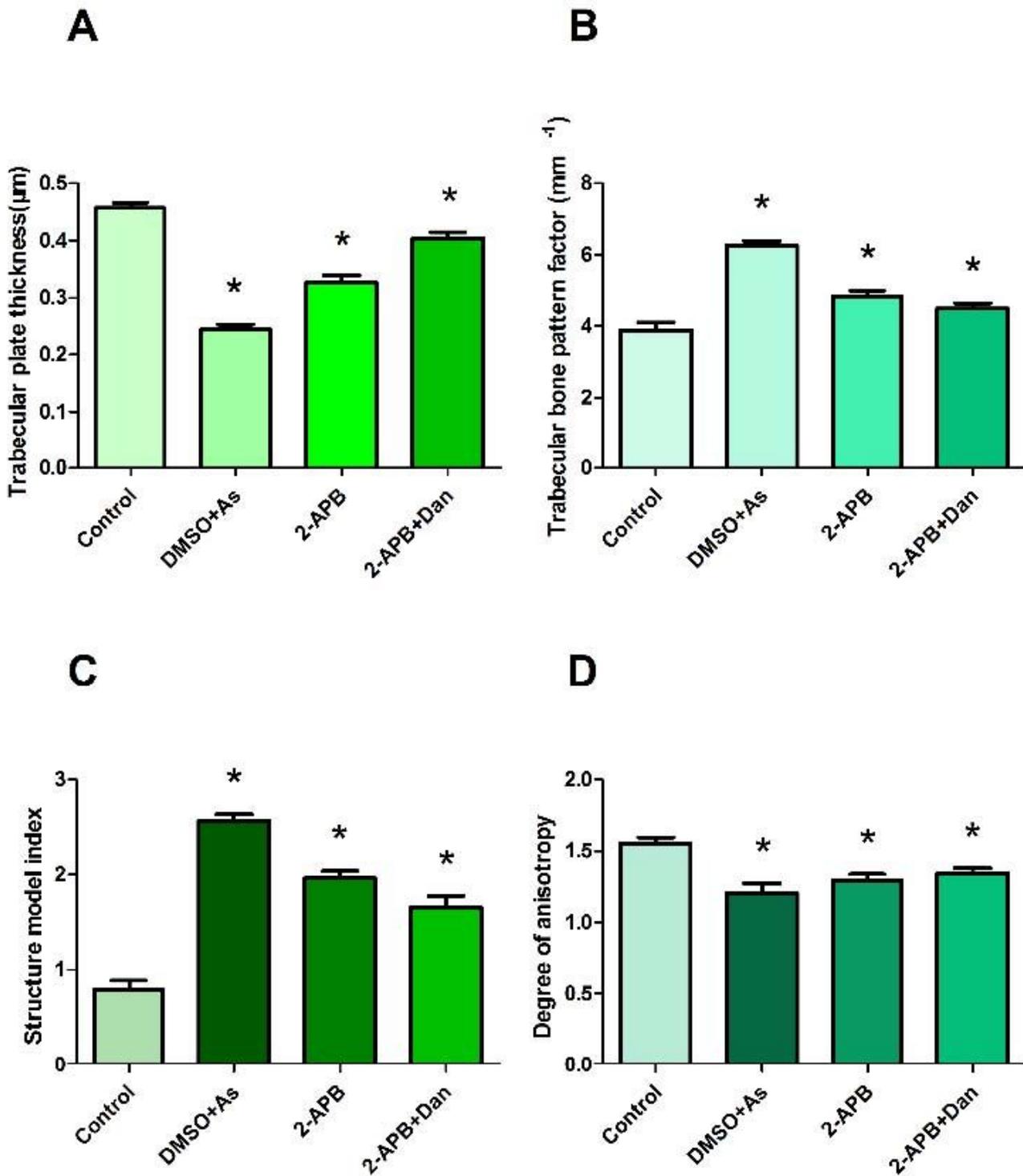


Figure 4

Bone metrological analysis A: Tb.Th; B: TBpf; C: SMI; D: DA * Compared with the control group, $P < 0.05$.

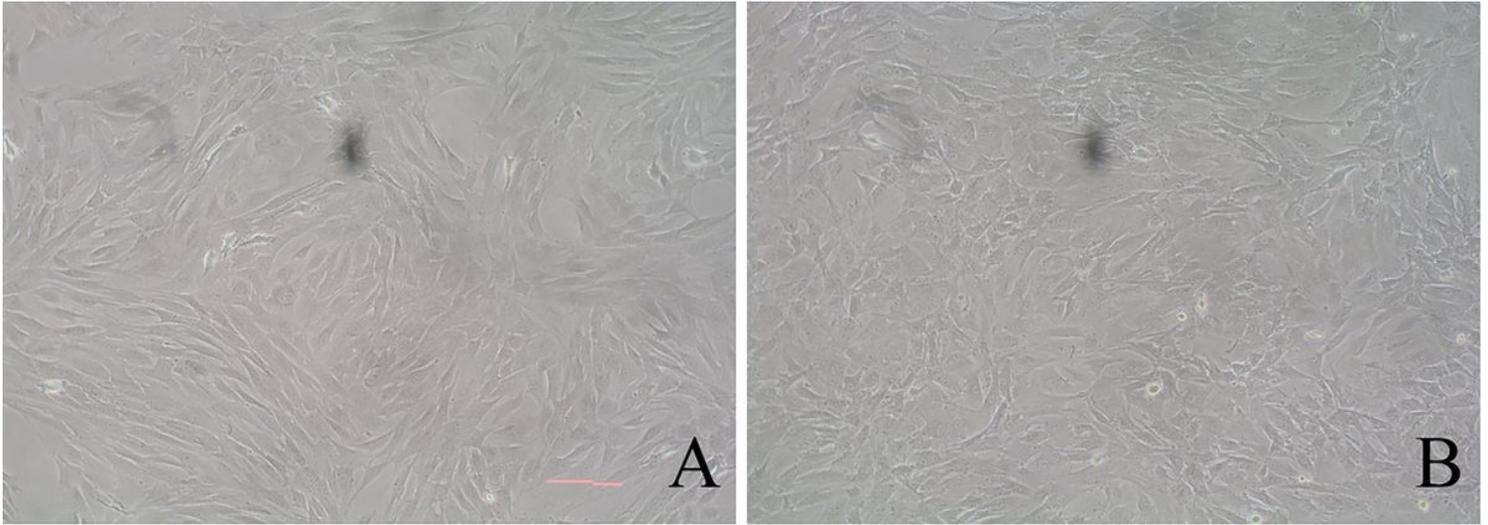


Figure 5

Primary culture of BMSCs A.B Third generation BMSCs (×100)

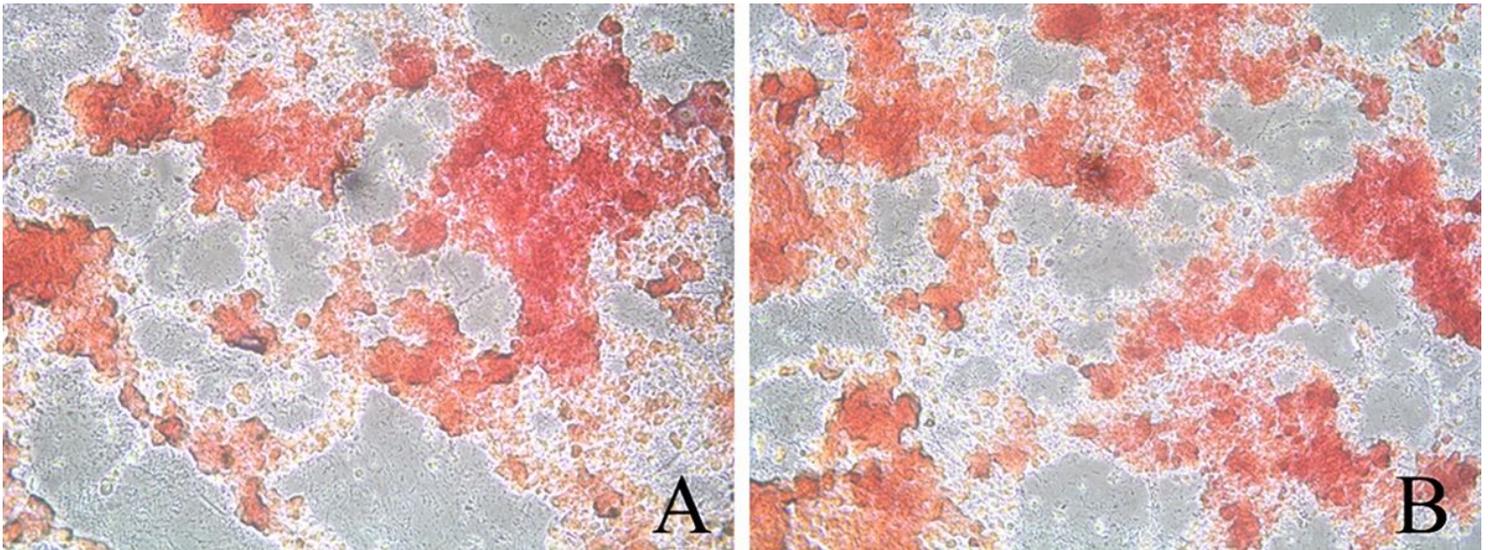


Figure 6

Osteogenic differentiation of BMSCs A.B Osteoblasts differentiated from third generation BMSCs (× 100)

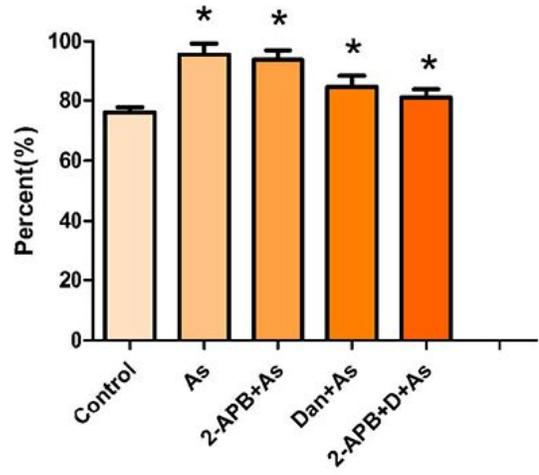
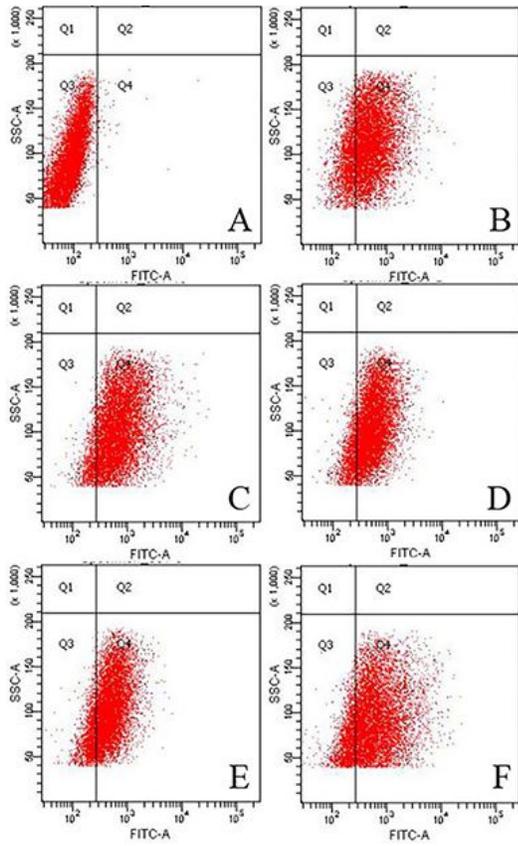


Figure 7

Ca²⁺ concentration of BMSCs A: blank group; B: control group; C: arsenic poisoning group; D: dantrolene intervention after arsenic exposure; E: 2-APB intervention after arsenic exposure; F: 2-APB + dantrolene combined intervention after arsenic exposure.

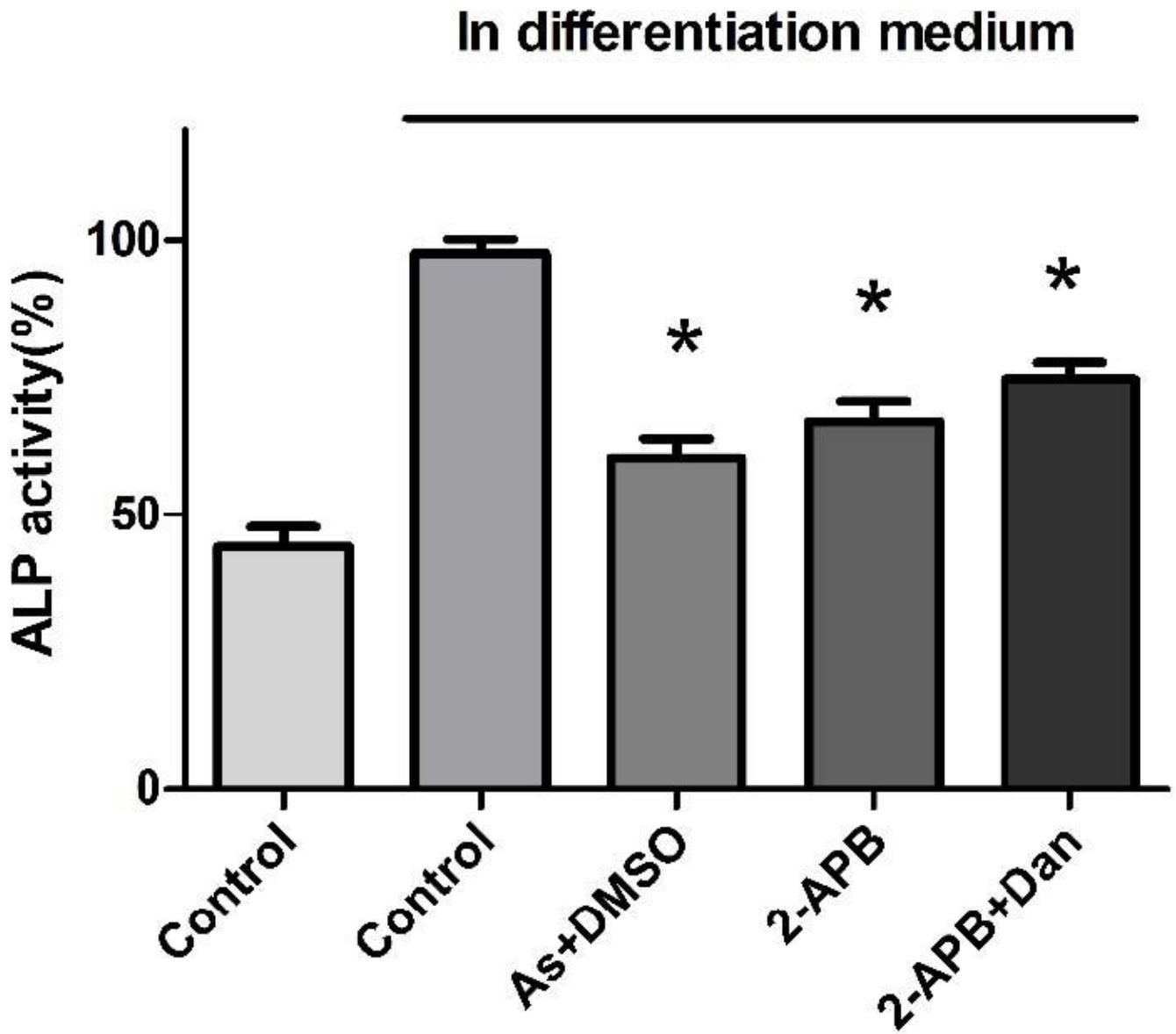


Figure 8

ALP activity of BMSCs * Compared with the control group, $P < 0.05$.