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Rescuing fluoride-induced skeletal injury in rabbits by increasing the proportion of protein and calcium in the diet

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ABSTRACT

Purpose: Fluoride(F) is ubiquitous in the environment, and excessive F intake can lead to skeletal injury. Currently, medical measures against F are not applicable for long-term protection in areas with excessive F levels. This study aims to investigate whether adding protein and calcium to the daily diet can rescue skeletal injury and skeletal mineral composition in rabbits exposed to excessive F.

Methods: Network toxicology was used to analyze the skeletal toxicity of F. X-ray detection is used to detect skeletal conditions, Hematoxylin and Eosin (H&E) staining and scanning electron microscopy (SEM) are used to evaluate skeletal ultrastructure, X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) technologies were employed to detect bone crystal and mineral content.

Results: The experimental results suggest that HiF group exhibited characteristic signs of fluorosis, including an enlarged bone trabecular meshwork, reduced bone density, and a disorganized trabecular arrangement. After increasing the protein and calcium content in the feed, the bone trabecular meshwork was restored in the rabbit femurs, with bone density and trabecular number returning to normal.

Conclusions: In summary, increasing the protein and calcium content in the diet can mitigate F-induced skeletal injury. This is a feasible and effective solution for regions with poor medical conditions and high incidence of fluorosis.

Keywords: *Skeletal injury; Protein; Calcium; Fluorosis; osteoporosis*

INTRODUCTION

Fluoride (F) is an essential element for humans and animals. It is widely distributed in nature and can enter the body through ingestion, contact, and inhalation.[1] Appropriate F intake promotes the absorption of calcium and phosphorus in the human body, facilitating the formation and development of bones and teeth.[2] However, in some areas with excessive F levels, F becomes a threat to the health of local people and animals.[3] Globally, more than 15% of regions have experienced problems with excessive F levels, with some Asian countries being the most severely affected.[4, 5] According to a report by the Chinese Center for Disease Control and Prevention, 1041 areas with F-induced drinking water-related diseases have been monitored in 28 provinces across the country, most of which have now been brought under control.[6] But fluorosis remains a severe public health problem in many countries, such as India, Ethiopia, etc..[7] Furthermore, the incidence and severity of chronic fluorosis depend on F concentration and an individual's absorptive capacity and nutritional status.[8] It is becoming increasingly clear that the degree of F toxicity varies considerably across different regions. The high incidence of fluorosis tends to be in areas with the most irrational diets and the worst nutrition.[9, 10] Therefore, dietary nutrient supplementation was aimed as a possible therapeutic option against fluorosis in areas with excessive F. Previous studies have shown that the toxicity of F can be effectively reduced by adding antioxidants, protein, and calcium to the diet.[11, 12] Unfortunately, feed usually needs to be stored for

several months, or sometimes even a year. Adding antioxidants to feed does not guarantee that it will remain unspoiled for such long periods. Therefore, improving the dietary composition of feed is considered a simple and feasible method of intervening in F poisoning in most production cases.

Calcium, an essential nutrient for bones, promotes bone resorption and regeneration. Our previous study demonstrated that calcium significantly alleviated F-induced endoplasmic reticulum stress and mitochondrial dysfunction in the skeletal system, as well as an abnormal proliferation of osteoblasts.[13] However, low calcium levels can exacerbate fluorosis and increase bone loss due to decreased mineralized trabecular and cortical bone volume.[14] Therefore, it is crucial to make a reasonable determination of the calcium dose in the animal diet to alleviate fluorosis. In addition, protein is vital for bone strength and helps with bone healing.[15] The lack of protein diets aggravates F damage to the body, and rich protein promotes the reduction of F toxicity.[16, 17] Therefore, it is imperative to add a high-protein diet to the diets of fluorosis animals. However, there are few studies on the protective effects of protein and calcium supplementation on collagen fiber formation and mineral crystal structure of bone under low-nutrient conditions, especially after fluorosis. Therefore, the present study established a rabbit model of protein and calcium supplementation under conditions of low nutrition and high F. The effects of protein and calcium supplementation on bone density, bone tissue morphology and ultrastructure, crystal structure, and mineral content in fluorosis rabbits were examined by X-ray, hematoxylin and eosin staining (H&E), scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR) techniques. This study provides new insights into the mechanisms of protein and calcium as dietary supplements to mitigate F-induced skeletal injury and provides information for their future development as therapeutic agents and functional foods. In addition, since many diseases in rabbits have similar mechanisms to those in humans, such as chondrodysplasia and hereditary osteoporosis, the research results can be more applicable to humans.

MATERIAL AND METHODS

Network toxicology analysis

Network toxicology was used to analyze the potential target genes induced by F and skeletal injury. The keywords “F” and “skeletal injury” were entered into the CTD(https://ctdbase.org/) and GC(https://www.genecards.org/), respectively. For searching related genes. The relevant genes were obtained, and enriched analysis was performed on the website(http://www.bioinformatics.com.cn/). In addition, the above genes were intersected and protein interaction analysis was performed on the string database (https://cn.string-db.org/). The protein interaction data that was obtained was then visualized and analyzed using Cytoscape (version 3.10.3).

Table 1. The ratio (%) of ingredients added to the basal diets

Ingredients	Low nutrition feed formula Ⅰ	Low nutrition feed formula Ⅱ	Low nutrition supplement protein formula Ⅲ	Low nutrition supplement calcium formula Ⅳ
Corn stover	25.00	25.00	30.40	21.00
Flour	16.00	16.00	12.00	17.00
Wheat bran	11.00	11.00	9.00	10.00
Soybean meal	-	-	24.00	-
The soybean straw	39.00	39.00	20.00	30.60
Rice bran	8.00	8.00	4.00	16.00
Calcium hydrogen	-	-	-	2.80
Stone powder	0.20	0.20	0.36	2.25
Salt	0.50	0.50	0.50	0.50
Sodium F	-	4.42×10^{-2}	4.42×10^{-2}	4.42×10^{-2}

There was no sodium F in formula Ⅰ, and 442 mg/kg sodium F (200 mg/kg F^-) was added in formulas Ⅱ, Ⅲ, and Ⅳ.

Table 2. Nutrient levels of the rabbits' diets in each group

	Malnutrition control (MC)	High F (HiF)	High F and high protein (HiF + HiPr)	High F and high calcium (HiF + HiCa)
F^- (mg/kg)	20.10	200.00	200.00	200.00
CP (%)	8.58	8.58	18.41	8.35
Ca (%)	0.49	0.49	0.46	2.23
P (%)	0.24	0.24	0.26	1.33
DM (%)	88.00	88.00	88.00	89.00
DE (MJ/kg)	9.48	9.48	10.37	9.48

CP: Crude protein; Ca: Calcium in diet; P: Phosphate; DM: Dry matter; DE: Energy density

Group MC, HiF, HiF+HiPr, and HiF+HiCa correspond to the added rabbit feed formula Ⅰ, Ⅱ, Ⅲ, and Ⅳ. A standard rabbit diet contains 14 % (140 g/kg diets) protein and 1 - 1.5 % (10-15 g/kg diets) Ca (see GB 14924.3-2010 Laboratory animals-Nutrients for formula feeds).

Animals and experimental groups

A total of 80 clinically healthy weaned (1-month-old) New Zealand rabbits (half male and half female) weighing 1.07 ± 0.25 kg were housed in ventilated animal rooms (22-25 °C temperature, 55 ± 5% humidity, 12 h light/dark cycle). All animal care and experimental procedures were approved by the Experimental Animal Ethics Committee of Shanxi Agricultural University (permit number: SXAU-EAW-2021Ra.KH.004003025) and strictly conformed to the Guide for the Care and Use of Laboratory Animals of Shanxi Agricultural University.

All rabbits were manipulated by a particular person and had free access to fresh water and standard chewing pellets for rodents. The rabbits were randomly allotted into four groups according to their weight (20 in each). The malnutrition control group (MC), the high F group (HiF), the high F and high protein group (HiF + HiPr), and the high F and high calcium group (HiF + HiCa) were fed pellet diets based on formulas Ⅰ, Ⅱ, Ⅲ, and Ⅳ in Table 1. The calcium-phosphorus ratio in each group was 1.5 - 2.1, and all drank deionized water. The supplemental level of sodium F (NaF) in all F intake groups was 200 mg/kg (in terms of F ions, BioXtra, ≥ 99%, S7920, Sigma-Aldrich, USA), and the experiment lasted for 120 days. Groups and the levels of F^-, protein, calcium, and energy density in groups are shown in Table 2 by Wang et al.[17]

Sample collection and processing

On the 120th day of the experiment, randomly selected rabbits were anesthetized with pentobarbital sodium (30 mg/kg, intraperitoneally), weighed, and slaughtered (fasted the day before the experiment). The femur of the slaughtered experimental rabbits was excised, and the surrounding muscle tissue was removed. Femurs from one side of 6 rabbits were fixed in 10% formaldehyde and 2.5% glutaraldehyde, respectively, and stored at 4 °C for H&E staining and SEM. Femurs from one side of 2 other rabbits were rapidly encapsulated and frozen for X-ray examination. Bone samples from the other side of 8 rabbits were partially ashed in a muffle furnace (Beijing Liuyi Instrument Factory) at 550 °C after removing the middle marrow to determine fluorine, calcium, and phosphorus content in bone crystal samples. The remaining portion of the specimens was then ashed in a muffle furnace at 900 °C to further determine skeletal crystal composition by XRD and FTIR.

Body weight and femur length

Rabbit's body weight and femur length were measured at 30, 60, 90, and 120 days, respectively.

X-ray detection

After removing the surrounding soft tissue, the frozen femur samples were immediately transported to the National Experimental Teaching Demonstration Center of Animal Medicine of Shanxi Agricultural University for X-ray (Medical X-ray Machine, Italy) photography and observation.

Histopathological analysis

Bone samples were fixed in 10% formaldehyde solution at 4 ℃ for 24 hr and then placed in EDTA-Na decalcifying solution, which was changed at two-day intervals and maintained for three months. dehydrated by gradient alcohol, transparented by xylene, soaked in the wax, and embedded. The embedded samples were sliced into 5 μm, and then Hematoxylin and Eosin (H&E) staining was performed.

Ultrastructural observation

Femurs were immediately washed three times with phosphate buffer (0.1 mol/L, pH 7.4) to remove the mucus component from the sample surface. The

samples were fixed overnight with 2.5% glutaraldehyde at 4 ℃, washed with phosphate buffer, and dehydrated through a gradient of tert-butanol. The specimens were glued to the copper platform with double-sided conductive tape and sprayed with gold. The morphological features of the bone surface were observed and photographed with a JSM-35CF scanning electron microscope (Japan Electronics Corporation).

Measurement of F, calcium, and phosphorus content in skeletal crystals

The bone samples were ashed in a muffle furnace at 550 ℃ for 48 hours and ground, and the ash powder obtained was dissolved with hydrochloric acid. Part of the liquid was adjusted to pH 8 - 9 with sodium hydroxide, and the volume was fixed to obtain the solution to be measured for fluorine content. Afterward, nitric acid was added, and the volume was fixed to obtain the solution to be measured for calcium and phosphorus content. The F ion selective electrode method was used to determine the F content in the solution (GB/T 21057-2007, inorganic chemical products fluorine content determination method). The F content of bone samples was calculated according to the regression equation of the standard curve. Determination of calcium content in bone samples by potassium permanganate titration. The decomposition solution was used to determine calcium content. The absorbance of the solution was measured at 420 nm with a 721 spectrophotometer, and the phosphorus content was calculated according to the absorbance.

X-ray diffraction (XRD) procedures for bone

XRD analyses were carried out with samples of bone crystals in the D/Max-RB XRD equipment (Rigaku, Japan) using Cu Kα radiation at a speed of 4°/min using a rear graphite monochromator in the 2θ range from 20° to 60°, operating at 30 kV and 20 mA with the Slit system of DS = SS-1, RS = 0.16 mm. Each group's component and content of samples were analyzed according to the obtained pattern.

Determination of the Fourier transform infrared spectroscopy (FTIR) of skeletal crystals

The above bone powder ashed at a high temperature of 900 ℃ for 24 h in a muffle furnace was mixed and ground with potassium bromide at a ratio of 1:200, vacuumed under pressure, and the selected films were visually inspected for uniformity and the presence of visible particles. The samples were analyzed by FTIR using an infrared spectrometer (IR 200 spectrometer, Thermo Electron Corporation).

Statistical analysis

All data are expressed as mean ± SD. Comparison between groups was performed by one-way ANOVA and Tukey's multiple comparison. $p < 0.05$ was statistically significant, and $p < 0.01$ was highly statistically significant.

RESULTS

Potential toxicity of F to bone tissue based on network toxicology.

Using the Comparative Toxicogenomics Database (CTD) and GeneCards (GC) databases to assess the potential targets of F on genes associated with Skeletal injury. In the CTD database, 2554 genes were identified as potential targets of F, while 11372 genes were identified as being associated with Skeletal injury. There were 1591 overlapping genes between the two groups. To ensure the accuracy of the results, this study further conducted target gene mining in the GeneCards database. The results showed that 1170 genes were identified as potential targets of F, and 1158 genes were identified as being associated with Skeletal injury. There were 474 overlapping genes between the two groups. However, in the CTD and GC databases, there were 178 overlapping genes associated with F and Skeletal injury. The number of genes overlapping between the two databases indicates that there are numerous potential targets for F-induced skeletal diseases, and that bone tissue health is highly sensitive to F (Figure 1A). To further obtain potential key targets for Skeletal injury caused by exposure to F, this study conducted a protein-protein interaction network (PPI) analysis on the above-mentioned intersection targets, setting the highest confidence level (0.700), and obtained a total of 174 nodes and 1176 edges, with an average node degree value of 13.5. Then performed visualization analysis using Cytoscape, identifying highly related gene targets such as TNF, IL-6, IL-1β, AKT1, and Trp53 (Figure 1B). In order to elucidate the potential functions and enrichment pathways of F exposure-induced Skeletal injury, this study conducted a functional analysis of potential targets. Gene ontology (GO) enrichment analysis showed that, in terms of biological processes(BP), these genes were mainly related to ossification, oxidative stress response, and response to external stimuli. For cellular component (CC), these genes were mainly located in the cell membrane. For molecular function (MF), cytokine activity, antioxidant activity, and signal receptor activity were the main focus (Figure 1C). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that the above genes were mainly involved in the PI3K-Akt signaling pathway, TNF signaling pathway, MAPK signaling pathway, FoxO signaling pathway, IL-17 signaling pathway, etc., and 27 genes were enriched in cell apoptosis (Figure 1D). Therefore, the above-mentioned pathways and cell apoptosis should be focused on as they are related to tissue damage.

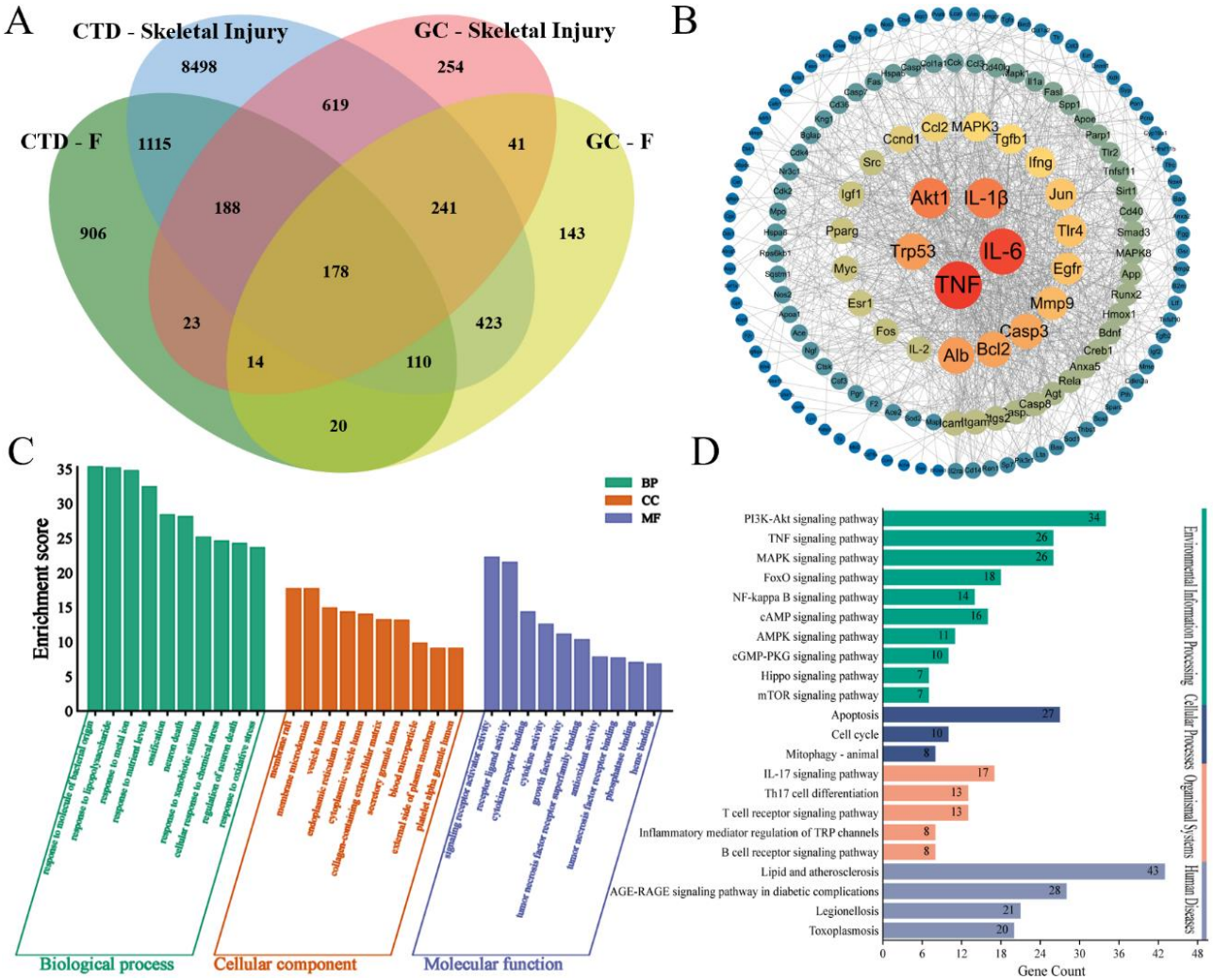


Figure 1. Results of network toxicology. **(A)** Venn diagram of F-induced skeletal injury. **(B)** PPI network diagram of F induced skeletal injury related gene. **(C)** Top 30 terms in the GO enrichment analysis. **(D)** Top 20 pathways of the target genes in the KEGG enrichment analysis.

Adding protein can restore F-induced inhibition of normal weight and skeletal growth in rabbits.

The experiment selected New Zealand rabbits that were similar in body weight and good health. During the laboratory process, the test results showed that the body weight of the HiF group decreased significantly compared to the MC group on days 60 and 120 ($p < 0.05$) and highly significantly on day 90 ($p < 0.01$). The HiF+HiPr group showed a recovery in body weight compared to the HiF group, with significant differences observed on day 30 and day 60($p < 0.05$) and highly significant differences on day 90 and day 120 ($p < 0.01$). Remarkably, the HiF+HiCa group didn't show statistically significant differences in body weight compared to the HiF group (Figure 2A). Compared with the MC group, the femur length of rabbits in the HiF group significantly shortened on 60, 90, and 120 days ($p < 0.05$). However, compared with the HiF group, the femur length of the HiF+HiPr group was restored, with significant changes observed on day 30, day 60 ($p < 0.05$), and highly significant differences on day 90 and day 120($p < 0.01$). However, the HiF+HiCa group only showed significant differences compared to the HiF group on day 30($p < 0.05$), and there was a subsequent trend toward recovery that was not significant.

X-ray examination of the femurs of rabbits treated with F for 60 days revealed that the MC group exhibited a dense reticular structure of the femoral bone trabeculae, small and densely distributed bone spots, and a clear femoral cortical bone structure, all of which were consistent with normal bone structure. However, in the HiF group, the majority of the femur exhibited enlarged bone trabecular meshwork, interspersed with coarse bone spots, with significantly increased blurring and disorganization of the bone trabeculae. The cortical bone exhibited a layered appearance, reduced bone density, and enlarged bone marrow cavity, suggesting possible osteoporosis-like fluorosis. In the HiF+HiPr group, the femur is close to normal bone structure, with a mesh-like bone texture interspersed with coarse, fluffy bone spots, accompanied by thinning of the cortical bone. In the HiF+HiCa group, the cortical bone of the femur has coarse, fluffy bone spots, and the bone texture mesh is enlarged, suggesting mild

fluorosis (Figure 2C). After 120 days of F treatment, bone density was significantly higher than the control group, with disorganized bone texture orientation and the appearance of coarse bone spots. The HiF+HiCa group had bone density similar to the normal group, with clearer texture and consistent orientation. The HiF+HiCa group had significantly higher bone density than other groups, with unclear texture and the appearance of coarse bone spots (Figure 2D).

In summary, a F concentration of 200 mg/L can cause abnormal weight loss, decreased bone density, and significant changes in the bone marrow cavity in rabbits. In addition, a high-protein diet can effectively prevent such defects caused by F.

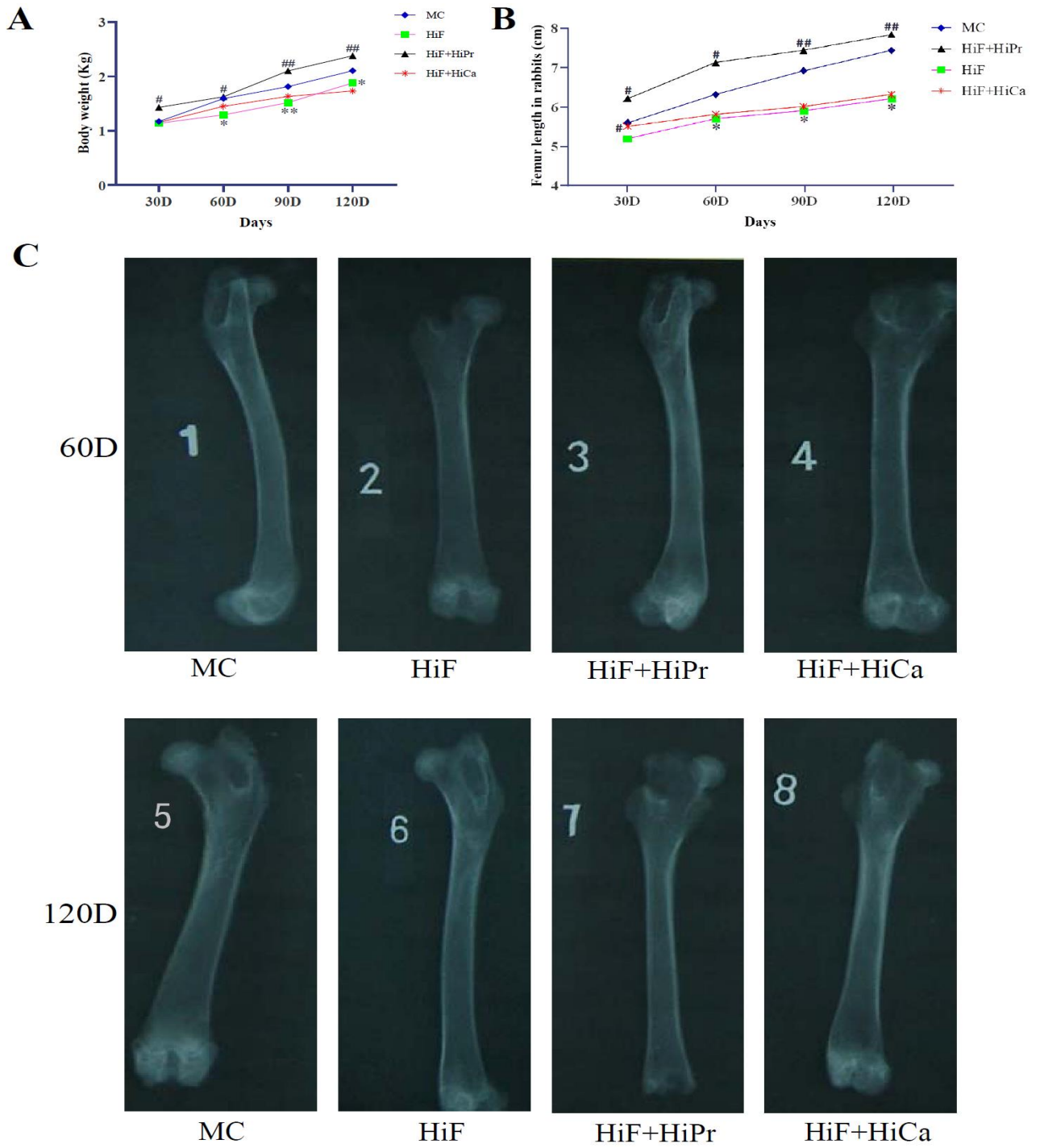


Figure 2. Effects of protein and calcium supplementation on F-induced body weight and skeletal injury in rabbits. **(A)** Body weight data were collected at 30, 60, 90, and 120 days of age (n=8). **(B)** Femur length was analyzed at 30, 60, 90, and 120 days in every group (n = 8). *p < 0.05, **p < 0.01, as compared with the MC group. #p < 0.05, ##p < 0.01, as compared with the HiF group. **(C)** X-ray observation of protein and calcium supplementation on F-induced skeletal injury in rabbits at days 60 and 120.

F-induced damage to the microstructure of rabbit femurs and effective intervention with proteins

H&E staining results showed that the trabecular bone in the MC group was neatly arranged, uniform in thickness, regular in shape, branched, densely arranged, evenly distributed, and cord-like. Bone cells were clearly visible in the trabecular bone, evenly distributed, and predominantly osteoblasts. However, in the HiF group, trabeculae were reduced, thickened, irregular, deformed, and broken. In this group, the reticular structure disappeared, the arrangement was disordered, and the distribution was irregular. Fragmented cavities of varying sizes can be seen, and the cords of the bone trabeculae have all disappeared. Bone cells decreased sharply, sporadically visible, disarranged distribution; the number of osteoblasts decreased, osteoclasts increased, and they are unevenly distributed on the trabecular bone. The number of bone trabeculae was raised in the HiF + HiPr group compared to that in the HiF group. The arrangement was neat and orderly, showing a reticular structure. Smaller numbers but with a larger size of osteocytes on trabecular bone, uneven distribution, sporadic visibility, and reduced number of osteoclasts were recorded in the HiF + HiPr group. Compared with the HiF group, the tissue structure in the HiF + HiCa group was chaotic, and the reticular system was damaged. Meanwhile, the trabecular bone was irregular in shape, and irregular deposition of calcium salts was seen in the broken cavity of the HiF + HiCa group than in the HiF group (Figure 3A). In the MC group, the collagen fibers were arranged neatly and tightly bundled; the arrangement was regular, and there was no breakage. However, compared with the MC group, the collagen fibers have entirely broken, shortening, disordered arrangement, and decreased content of collagen has caused cracks in the bone density. Here, the bone trabeculae on the inner surface of the bone density were also severely broken, and the bone resorption on the surface of the bone density is peak-shaped in the HiF group. Compared with the HiF group, the bone-dense surface showed a more extensive range of bone absorption; collagen fibers disappeared, fractured, and shortened, the surface was uneven, and inorganic substances were scattered on the bone surface in the HiF + HiPr group. In the HiF + HiCa group, there was no significant change in the bone surface, collagen fibers were utterly broken, and inorganic salts in the bone mass accumulated on the bone surface compared to the HiF group. The bone resorption on the bone surface was a honeycomb (Figure 3B). In terms of counteracting the destructive effects of F on bone microstructure, protein supplementation is more effective than calcium supplementation.

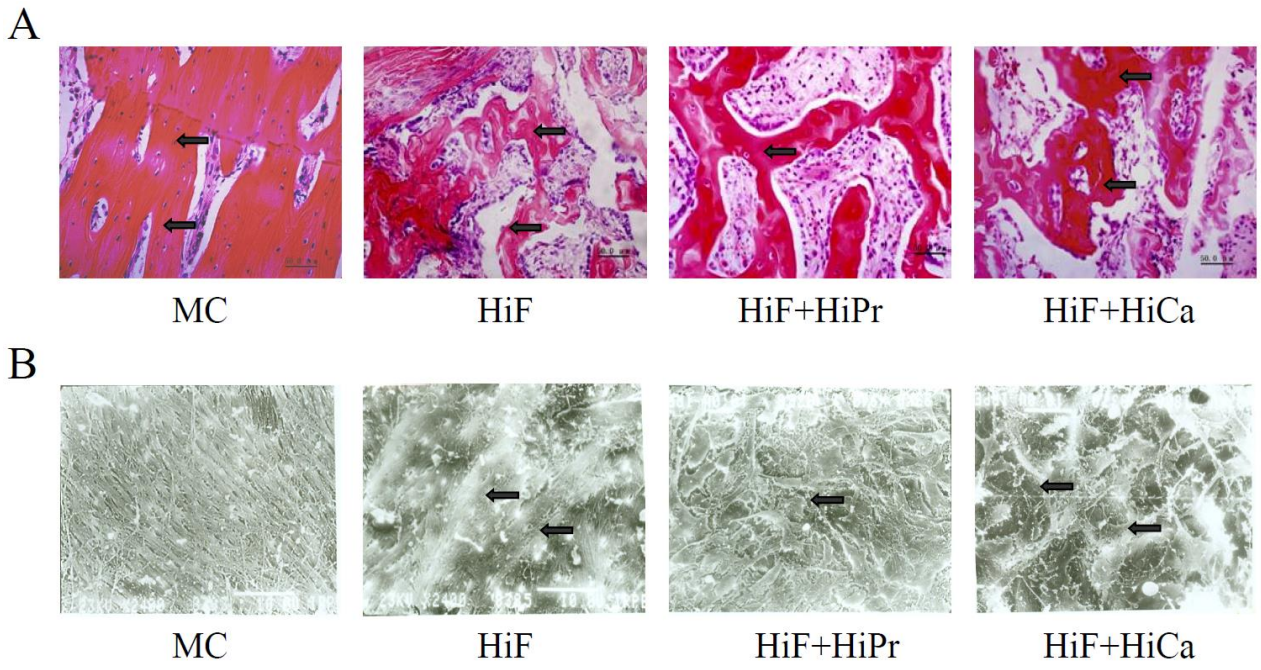


Figure 3. HE staining results and scanning electron microscopy ultrastructural observations of rabbit femurs. (**A)** The results of HE staining. Black arrows indicate the bone trabeculae. The trabeculae indicated by the arrows are all in an orderly arrangement in the MC group; the trabeculae in the HiF group are disordered, broken, and disappear; compared with the HiF group, the number of bone trabeculae in the HiF + HiPr group was increased and arranged in order; the HiF + HiCa group is similar to the HiF group, most trabeculae are fractured and absent. **(B)** Observation of protein and calcium supplementation on the ultrastructure of rabbit cortical bone induced by F. SEM was used to observe the ultrastructure alterations and cortical bone injuries(2400×). In the pictures, black arrows indicate the areas of broken, shortened, and disordered collagen fibers in the femoral cortex of rabbits.

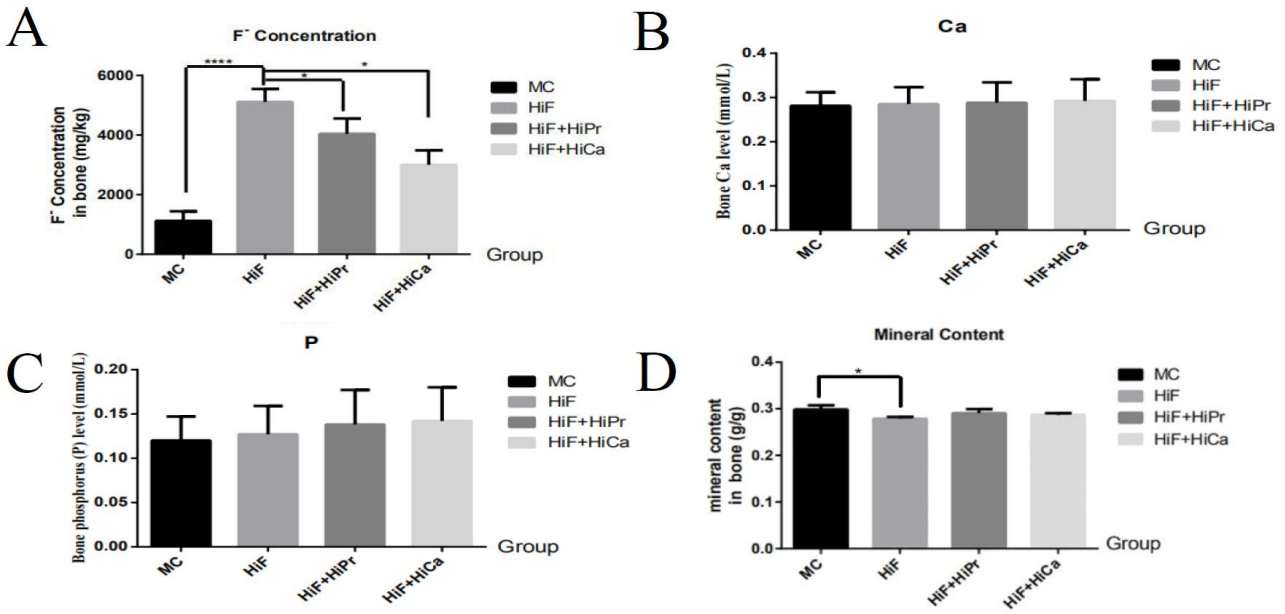


Fig 4. Effects of protein and calcium supplementation on F-induced bone F ion (**A), Calcium (B), phosphorus (C), and bone mineral content in rabbits(D).** Bone Ca, P, F, and bone mineral content in rabbits of each group are depicted as mean ± SD (n=8). *p < 0.05, ****p < 0.0001, as compared with the HiF group.

The F, Ca, P, and inorganic mineral content of rabbit femurs showed differences between the experimental groups.

The F deposition in the femurs of rabbits in the HiF group was significantly higher than that in the MC group ($p<0.01$), while the F deposition in the HiF+HiPr and HiF+HiCa groups was significantly reduced compared to the HiF group ($p<0.01$) (Figure 4A). The calcium and phosphorus content in the HiF group showed an upward trend, but the difference was not significant compared to the MC group. The calcium and phosphorus content in the HiF+HiPr group and HiF+HiCa group did not show significant changes ($p > 0.05$) (Figure4B, C). The mineral content of the femur in the HiF group was significantly higher than that in the MC group ($p < 0.05$), while the HiF+HiPr group and HiF+HiCa group showed no significant changes compared to the HiF group ($p > 0.05$) (Figure 4D).

Analysis of rabbit bone crystal structure by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR)

The D/Max-RBX diffractometer and copper target were used to determine the crystalline structure of rabbit bones in this study. The X-ray diffraction patterns obtained indicate that the HiF group has the highest spectral resolution and sharp peaks. When compared with the standard spectra, the spectra of all four groups of bone samples in this experiment exhibit $Ca_{10}(PO_4)_5CO_3(OH)F$, $Ca_9HPO_4(PO_4)_5OH$, $Ca_5(PO_4)_3(OH)$, $Ca_3(PO_4)_2$, $Ca_{10}(PO_4)_6(OH)_2$, $Ca_{10}(PO_4)_6(OH)F$, $Ca_{10}(PO_4)_6F_2$, $Na_6Ca_4F_2(SO_4)_6$, $Ca_{10}(SiO_4)_3(SO_4)_3F_2$, and $Ca_8H_2(PO_4)_6$. Among these apatites, all except $Ca_3(PO_4)_2$ belong to the hexagonal crystal system. In the MC group, the content of $Ca_{10}(PO_4)_6(OH)_2$ was higher compared to other groups, while the content of $Na_6Ca_4F_2(SO_4)_6$ was significantly reduced in the HiF+HiCa group. Other components showed no significant changes. Surprisingly, the results of the HiF+HiPr group indicated that supplemental protein did not improve the crystal structure of rabbit bones (Figure 5A).

The infrared spectrum (FTIR) consists of spectral lines of PO_4^{3-}, CO_3^{2-}, OH^-, and H_2O (Figure 5B). The PO_4^{3-} v_4 absorption peak of the measured sample is a double peak with a large peak splitting width and a small splitting depth. v_1 may overlap v_3 in the form of an absorption shoulder. According to the comparison in the figure, the PO_4^{3-} absorption peaks of each group are basically consistent, with no significant changes. All bone hydroxyapatite CO_3^{2-} absorption peaks exhibited v_3 and v_2, with v_2 being weaker in intensity. The vibration frequency of v_3 varied depending on the position of CO_3^{2-} in the lattice. Blunt peaks were observed in all groups, suggesting that CO_3^{2-} may have occupied the position of PO_4^{3-}. There were no significant differences between groups. The stretching vibration band (~3550 mm^{-1}) of the OH^- absorption peak in the MC group is larger than other groups. The area of the OH^- absorption peak in the HiF + HiCa group is smaller than that in the HiF group. Potassium bromide absorbs water and produces an H_2O absorption peak stretching vibration band, but the absorption peak stretching vibration band of skeletal hydroxyapatite is stronger, which is not caused by potassium bromide absorbing water. The results show that there are no significant changes in each group.

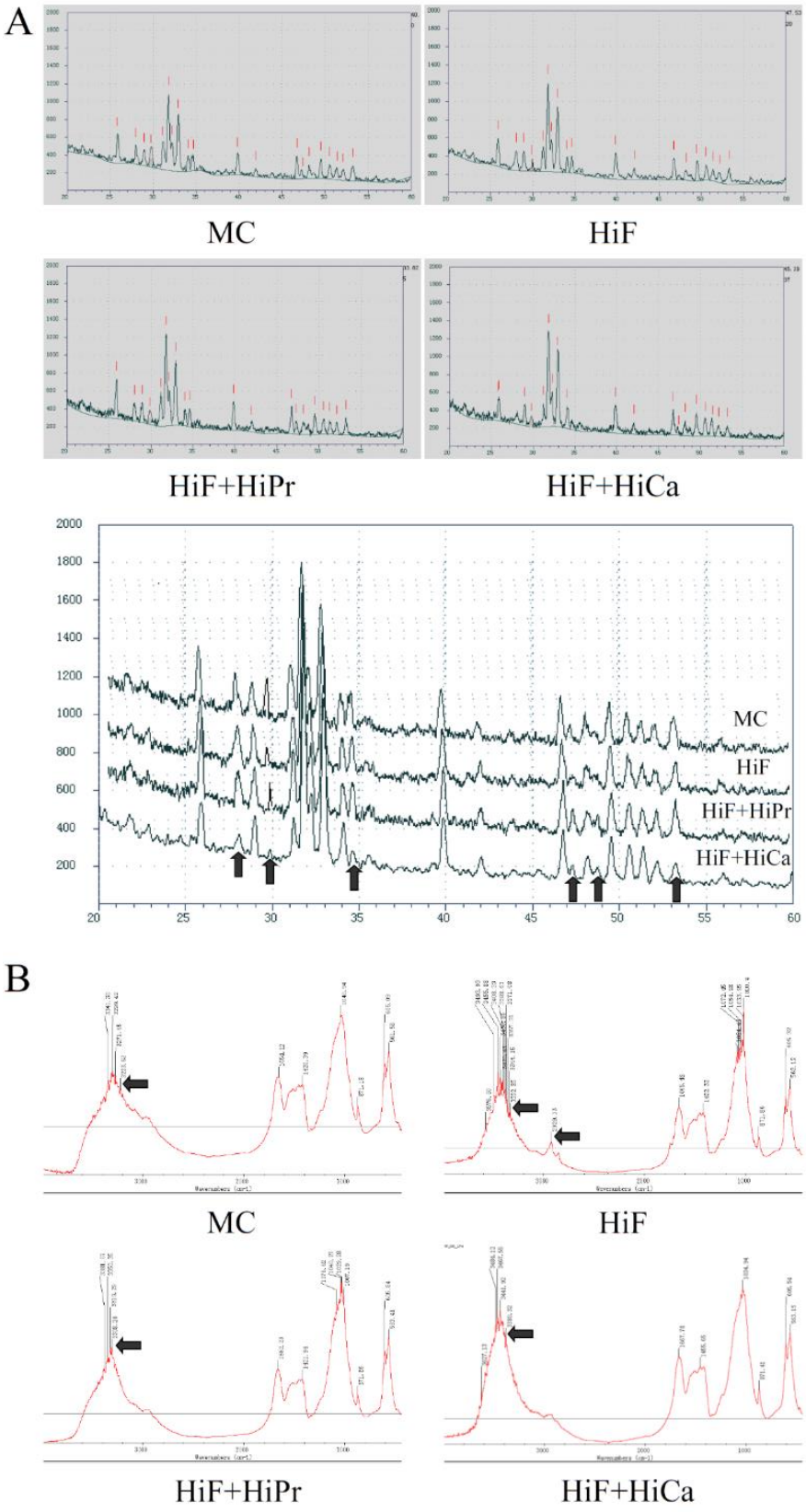
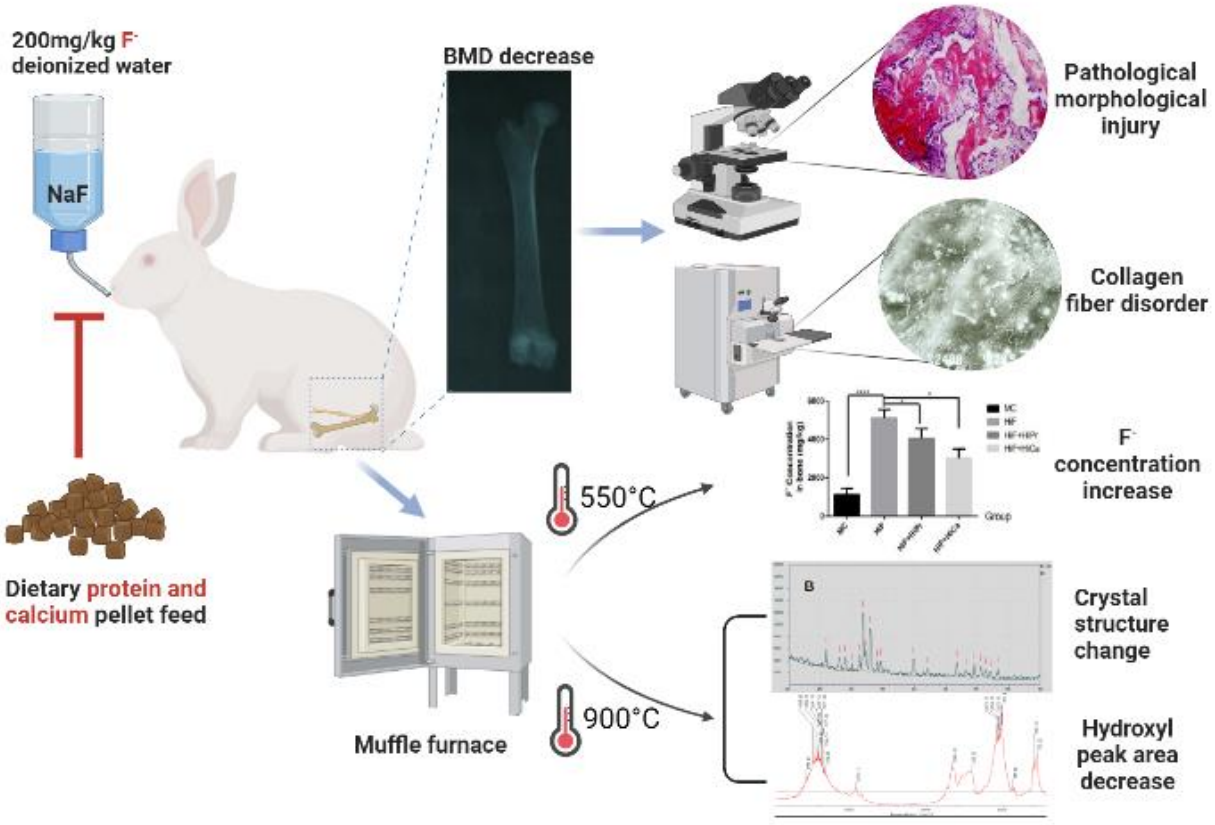


Figure 5. XRD and FTIR measurement of protein and calcium supplementation on F-induced femoral bioapatite in rabbits. **(A)** The Result of XRD measurements of each group, and the combination chart of each group, the black arrow marks the absorption peaks with differences between the groups. **(B)** FTIR measurement of protein and calcium supplementation on F-induced femoral bioapatite in rabbits. The absorption peaks were 3493-3332 cm^{-1} for OH^-, 1667-1654 cm^{-1} for H_2O bending vibration, 1455-1420 cm^{-1}, 1455-1420 cm-1 for CO_3^{2-} v_3 asymmetric stretching vibration, 1040-1007 cm^{-1} for PO_4^{3-} v_3 asymmetric stretching vibration, 872-871 cm^{-1} for CO_3^{2-} v_2 bending vibration, and 606-605 cm^{-1} and 564-561 cm^{-1} for PO_4^{3-} v_4 bending vibration. Black arrows indicate specific hydroxyl absorption peaks in the bone crystal.



Graphical Abstract

F at 200 mg/kg can cause damage to bone morphology and crystal structure in rabbits. Dietary protein attenuated the damage to bone morphology and collagen fiber structure in fluorosis rabbits. Dietary calcium attenuated bone F deposition in rabbits after F exposure. This image was created in BioRender(https://www.biorender.com/)

DISCUSSION

Numerous studies have reported that long-term exposure to F in animals and humans causes time-dependent damage to bones.[2,18] F causes skeletal damage through multiple pathways. This study used Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to find that the toxicity of F may be related to the PI3K/Akt, TNF, MAPK, FoxO, and mTOR pathways. It has been reported that the toxicity of F is related to the PI3K/Akt pathway.[19, 20] Baohong Zhao et al.[21] demonstrated that tumor necrosis factor (TNF) plays a key role in inflammatory bone resorption and related pathogenesis in diseases such as rheumatoid arthritis and periodontitis. Matthew B Greenblatt et al.[22] reported that the p38 MAPK pathway is essential for normal bone formation in mice. FoxO transcription factors suppress osteoclast generation by counteracting ROS production through upregulation of antioxidant enzymes.[23] Downregulation of mTOR leads to osteogenesis imperfecta.[24] As is well known, Oxidative stress can induce apoptosis,[25] autophagy,[26] pyroptosis,[27] inflammation,[28] and ferroptosis.[29] This involves changes in the expression levels of IL-1β, IL-6, Caspase-3, Bax, Bcl-2, etc., which is consistent with the network toxicology prediction in this study. Furthermore, F can cause oxidative stress and contribute to the apoptosis and autophagy of osteoblasts or osteoclasts, thereby causing damage to bone tissue.[30,31] Therefore, the mechanism of bone damage caused by F may largely stem from downstream reactions triggered by oxidative stress. Thus, the application of antioxidants may be an effective treatment strategy to alleviate F-induced bone damage. However, the strategy adopted in this study is to use proteins to prevent the body from absorbing F, thereby preventing bone damage at its source. Some scholars also believe that calcium can rescue damage caused by F.[11,24,31]

Mahmood Yousefi's research[32] found that F has no statistical significance with Body mass index(BMI). Other studies have shown that F has a significant negative effect on animal growth.[33,34] The results of this study show that high doses of F inhibit growth in rabbits and that supplementation with protein significantly increases their body weight. This indicates that protein supplements not only reduce the body's absorption of F,[35] but also provide essential nutrients. Although calcium can also reduce the body's absorption of F, it

cannot provide nutrients for the body's growth and has limited effectiveness against F. Fluorosis is one of the characteristic pathological changes of chronic F poisoning. Its pathogenesis involves excessive F entering the body and affecting calcium metabolism and bone formation processes. Cortical bone demineralization is an important manifestation of osteoporosis in fluorosis. Osteoporosis in fluorosis has previously been explained as resulting from excessive F doses inhibiting the formation of bone matrix.[36] If the calcium supply is adequate, blood calcium levels can be maintained at normal levels. However, if calcium supply is insufficient, it not only increases F absorption from the intestines but also disrupts calcium metabolism, leading to a negative calcium balance. The increased F^- /$Ca2^+$ ratio F deposition in bones and its toxic effects on the kidneys, further stimulate hyperparathyroidism, leading to osteoporosis and osteomalacia.[36] In fluorosis, the entire skeleton may be affected, with prominent damage to bone joints. The dual-energy X-ray absorptiometry technique revealed that bone density was decreased after F exposure.[37] Rakesh Ranjan[38] conducted a F concentration gradient experiment and used X-ray detection results to show that bone density and cortical bone thickness were significantly reduced. This study showed that bone density decreases in the early stages of F exposure, while bone density and cortical bone thickness increase with prolonged F exposure.

The results of dietary protein supplementation under high F conditions showed that the number of bone cells increased,[35] which alleviated the injury of the trabecular reticular structure in fluorosis. The microscopic structure of bones has a significant impact on bone strength. Fluoride can significantly alter bone mineral deposition and trabecular separation, leading to an increased risk of bone fractures. Increased calcium intake can alleviate this phenomenon.[19] However, in this study, the results of the calcium supplement showed no significant improvement in the bone trabecular network structure, which may be due to the irregular calcium salt deposition of increased bone mass, which ultimately led to deformities in the trabecular morphology.[13] In addition, protein supplementation increased the collagen content and the degree of cross-linking, resulting in the toxicity of fluorine and providing an excellent raw material for collagen synthesis. Many scholars have observed bone tissue samples from patients with fluorosis using scanning electron microscopy(SEM) and have reached a consistent conclusion: the trabeculae are irregularly arranged, the collagen fibers are disordered in their orientation, and they were vary in thickness, and are even prone to dissolution and fracture.[39-41] Consistent with the findings of this study.

In this experiment, we found that although bone F content was significantly increased after F exposure, bone calcium and bone phosphorus content were not significantly different between the statuses. This suggests that the increase in bone F content primarily exists in the form of fluorapatite, while calcium F (CaF_2) constitutes a minor portion of minerals in bones. After supplementing with protein and calcium, there were also no significant changes in bone calcium and phosphorus levels. This suggests that despite adequate calcium intake in the diet, the amount of calcium deposited in bones is not significant; While protein supplementation increases the raw materials for the skeletal framework structure, calcium, phosphorus, and other minerals can only be deposited in their respective positions after the skeletal framework structure has stabilized, thereby maintaining the skeletal structure's biological and mechanical integrity. Under low-nutrient and high-F conditions, dietary calcium supplementation can reduce F deposition in bone and prevent significant changes in blood calcium caused by parathyroid dysfunction, reducing calcium mobilization in bone. However, long-term excessive calcium supplementation is ineffective in relieving skeletal fluorosis. It is possible that the protein does not reach the amount required for bone tissue synthesis and metabolism, resulting in a lack of calcium function.[42]

X-ray diffraction is reported to elucidate the mineralogical properties of bone components accurately. FTIR spectroscopy is considered a semi-quantitative tool for characterizing molecular bond vibrations, as each spectrum is a chemical fingerprint for mineral identification. These two techniques can be used to analyze unique information about the crystal structure of minerals.[43,44] Calcium supplementation increased the calcium content in the crystals, improved the calcium-phosphorus ratio, and significantly increased the peak area of hydroxyl groups. The results for $Ca_9HPO_4(PO4)_5OH$ and $Ca_3(PO_4)_2$ are the same as those for Hydroxyapatite (HAP) is the same result as this study, presumably because HAP has the highest thermodynamic stability among calcium phosphate materials.[45] Therefore, most calcium phosphate materials tend to convert to HAP under certain conditions. However, in the XRD results, the intensity of the diffraction peaks of $Ca_9HPO_4(PO_4)_5OH$ corresponding to the crystalline surface differed, probably due to the influence of other phases. The appearance of $Ca_{10}(PO_4)_5CO_3(OH)F$ indicates that there is CO_3^{2-} substitution of PO_4^{3-} in the crystal sample, and the diffraction peak of carbon hydroxyapatite is slightly broader than that of hydroxyapatite, indicating that the substitution of carbonate ions has caused a change in the lattice and a decrease in the crystallinity of carbon hydroxyapatite46. The CO_3^{2-} spectra of all samples were free of sharp peaks, high and low bimodal peaks, indicating that carbon hydroxyapatite was present in the bones, so protein and calcium supplementation could not change the composition of the bone mineral. The formation of $Na_6Ca_4(SO_4)_6(OH)_2$ might be due to

the replacement of phosphate groups in apatite by sulfate groups, requiring partial replacement of calcium with monovalent cations such as sodium to compensate for the charge. However, due to the volatility of Na_2SO_4, $CaSO_4$, CaF_2, and $Ca_3(PO_4)_2$ undergo non-chemical reactions, resulting in little change in $Na_6Ca_4(SO_4)_6(OH)_2$ content.[46] Fluorosis aggravates osteoporosis and bone loss with a subsequent reduction in bone mineral content.[47] However, bone minerals consist of many amorphous mixtures of calcium and phosphorus,[48] and dietary supplementation with protein or calcium doesn't significantly change the total bone mineral content. Proteins and calcium alleviate bone damage by reducing F absorption, thereby reducing the destruction of the hydroxyapatite structure by F, In particular, Ca can form insoluble CaF_2 with F, preventing the absorption and bioavailability of F in the gastrointestinal tract. which significantly mitigates the negative effects of F.[49] The presence of F can increase the compressive strength of apatite crystals, enhance protein adsorption capacity, and improve cell adhesion capacity.[50] Therefore, protein supplementation will result in more protein being enriched in bones where F is present. In addition to being a major component of bones, calcium can also counteract damage caused by oxidative stress.[51] In summary, high-protein and high-calcium diet still has a certain positive effect on F-induced skeletal injury symptoms.

CONCLUSIONS

This study investigated the effect of dietary nutrition on rabbit bones, using protein and calcium as nutritional supplements to mitigate fluorosis toxicity. High consumption of drinking water with a F concentration (200 mg/kg, in terms of F ions) in rabbits led to growth retardation, skeletal injury morphology and ultrastructure, destroyed the typical crystal structure, and reduced the mineral content. Regarding pathomorphological bone alterations, dietary protein supplementation alleviates F-induced growth retardation, collagen fibril disruption, and cellular abnormalities. Dietary calcium supplementation was effective in reducing F deposition. In conclusion, high-protein and high-calcium diets have a positive effect on F-induced skeletal injury in malnutrition rabbits, and high-protein diet has a more significant alleviating effect.

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CONFLICT OF INTERESTS

None

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