# WHY DO PENGUINS NOT DEVELOP SKELETAL FLUOROSIS?

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**SUMMARY: Despite having an exceptionally high fluorine (F) concentration in their bones (up to 9000 µg/g in the present study), radiographs of mature Adélie penguins (*Pygoscelis adeliae*) do not show any symptoms of skeletal fluorosis. In this research, a series of chemical fractionation and speciation analyses for F gave a tentative explanation for this seemingly abnormal fact. The results showed that the inorganic fraction of F in penguin bones represented only about one-third of the total F with the rest bound organically, mostly in the form of fluorinated chitin or its derivatives. A laboratory experiment with rats on a high F intake indicated that chitin might prevent skeletal fluorosis by effectively combining with F and inhibiting abnormal mineralization, thereby decreasing the expected increase in bone mineral density.**

Keywords: Adélie penguins; Chitin; Organic fluoride; Penguin bones; Skeletal fluorosis.

## INTRODUCTION

Fluorine (F) as fluoride anion is ubiquitous in the environment and is normally present in bones and teeth, but excessive amounts can become toxic and lead to debilitating fluorosis in man and animals.[1-3] The average F concentration in bones of humans, cattle, swine, chickens, and birds (except penguins) varies from 500 to 1000 µg/g. A concentration between 3,500 and 5,500 µg/g in human bones increases bone mass and marks the preclinical phase of F-induced osteosclerosis.[4] The clinical manifestation of osteosclerosis includes bone and joint pain, spinal compression, progressive restricted movement of various joints, bone deformities, higher bone mineral density (BMD), and calcified ligaments.[5,6]

A previous study in our laboratory showed that although bones of Adélie penguins (*Pygoscelis adeliae*) have extremely high concentrations of F (up to 7187 µg/g in that study), their radiographs do not show any signs of skeletal fluorosis.[7] In the present study, we analyzed the F fractions and mineral composition of penguin bones, examined the effect of chitin on F metabolism in rats, and present a tentative explanation for the absence of fluorosis in the penguin bones.

## MATERIALS AND METHODS

*Sampling:* The sampling was performed during December 1998 to March 1999 near China's Zhongshan station (69°22'S, 76°24'E) in a coastal site of Prydz Bay, East Antarctica. Penguin and other seabirds such as skua (*C. maccormicki*) live

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near the station during the austral summer. An Adélie penguin colony with several large rookeries is located about 1 km east of the station at the sampling site described in detail by Xie and Sun.[7] A fresh skeleton (almost fleshless) of an adult Adélie penguin, which had died naturally, was collected, and the bone samples were pretreated using clean techniques (e.g., washed three times with deionized water), dried in an oven at 105ºC for 12 hr), and then kept at 4ºC until lab analysis. The tested rat bone F was determined according to above method.

*Fractionation analysis:* Since different bones of the penguin differ very little in F concentration,[7] for convenience, wing bones were chosen for this study. Wing bone samples (0.5~2.0 g) pretreated as noted above were dipped in boiling water to remove soft tissues, dried in an electrical furnace at 105ºC for 12 hr, and then powdered in a mechanical agate mortar. The powdered samples were kept in clean glass bottles. Total F, inorganic, and "organic" F were determined by a F ion selective electrode (ISE) as described by Xie and Sun,[7] Tankersley et al.,[8] and Li[9] with minor modifications. Briefly, to determine the concentration of inorganic F, such as $CaF_2$, $(Ca,X)_{10}[PO_4,HPO_4,CO_3]_6[OH,Y]_2$, 200 mg of dried pulverized bone powder was dissolved in 25 mL of 0.01 M HCl, and the mixture shaken for 30 min. The digested mixture was filtered into a 50-mL volumetric flask and diluted to 50 mL using 0.01 M HCl. A 5-mL portion of the diluted solution was transferred to a 50-mL flask, two drops of 0.2% alizarin red was added, and the solution was then adjusted to a purple color by 5% NaOH. Afterward, 20 mL of citrate sodium buffer was added, and the resulted solution was diluted to 100 mL by deionized water.

To determine the concentration of "organic" F, the residues after inorganic F extraction by 0.01 M HCl were placed in 20 mL of 20% $HNO_3$ (V/V), heated to about 60ºC on an electric hot plate, cooled, filtered, and the filtrate diluted to 50 mL with deionized water. To determine the amount of concentration of total F, the bone samples were fused with 17 M NaOH in Ni crucibles placed inside a muffle furnace with the temperature slowly raised to 600ºC. The temperature was kept at 600ºC for 30 min, and then the residue was dissolved in water on a hot plate. The Fe and Al compounds, which interfere with F analysis, were removed by adjusting the pH of the solution to 8.5 and filtering the suspension. The F concentration of the solution was then determined by the ISE.

*Chemical structure analysis:* To identify the structure characteristics of the "organic" F residue, powder X-ray diffraction (M18X, Japan), electric energy spectrometry (ESCALABMKII, UK), and Fourier-transform infrared spectroscopy (MAGNA 750, USA) analysis were performed in the Laboratory of Structure Analysis at the University of Science and Technology of China.

*Experimental animals and treatment:* According to the guidelines of the Committee on Care and Use of Experimental Animal Resources, 26 newly weaned Wistar rats were used in this study. The rats were fed with a standard diet, and were given drinking water with the pellets. The animals were maintained under proper temperature 23±2ºC, ventilation, RH 55±10%, 12-hr light/12-hr dark cycle, and hygienic conditions. The animals were randomly distributed into three groups:

a control group (n=9), a F-exposed group (n=7) and a F-exposed + chitin group (n=10). On the day of weaning of the pups (day 0), the tap water used for the F-exposed and F-exposed + chitin groups was replaced by 150 μg F/mL F solution and 150 μg F/g fluoride (from NaF) Sinopharm Group Chemical Reagent Co., LTD, Shanghai, China) + 0.3 g chitin/kg bw/day (Sinopharm Group Chemical Reagent Co., LTD, Shanghai, China), respectively. At the age of three months, the animals were used for specimen collection.

*Specimen collection:* After anesthetizing the animals, blood were drawn from heart using heparinized tubes and stored at –20ºC. At the same time, the right tibiae were removed from the legs of animals. All the bone specimens were stored at –70ºC to prevent decalcification. The rat bone F was determined according to the same method as for the penguin bone F.

*Bone mineral densitometry:* In order to assess the differences in bone mineral density (BMD), the rat bone specimens were placed horizontally on the sensor field of the dual-energy X-ray absorptiometer (DXA). The DXA images were transferred to a computer; and the intensities of three regions from each image were measured. The average of the three measurements was designated as the BMD value of the specimen.

*Statistical analysis:* Data were analyzed by one-way ANOVA and presented as the mean±SD for each group. Probabilities less than 0.05 were considered significant.

## RESULTS AND DISCUSSION

*Fluorine in penguin bone:* The inorganic, organic, and total F levels in the Adélie penguin bones are given in Table 1. The total F level is about 8 times greater than that in leg bones of skua, one of the seabirds living in the maritime Antarctic,[7] and it is far beyond the normal F concentrations of 500 to 1000 μg/g in bones of human beings and other mammals.[4]

**Table 1.** Concentrations (μg/g) of organic, inorganic, and total fluorine in wing bone and wing arthrosis bone of Adélie penguin

| Sample Code | Organic F | Inorganic F | Total F | Sample description |
|---|---|---|---|---|
| PB-01 | 5795 | 3197 | 9080 | Arthrosis part of wing bone |
| PB-02 | 5748 | 3311 | 9122 | Wing bone |

The abnormally high F concentration in Adélie penguin bones can be explained by the fact that about 81.79% of Adélie penguins' diet is krill, whose average body concentration of F is about 1,232 μg/g. The F concentration in the chitin crust of krill can be from 3,028~3,828 μg/g[10] to 5477 μg/g.[11] The radioisotope trace experiment by Dinman[12] verified that about half the F absorbed by animals is retained in the body, and 90% of the retained F is accumulated in the bones. The

high level of F in the penguin diet also leads to high levels of F in penguin droppings.[13]

To further investigate the exceptional tolerance of penguins to F, we determined the inorganic and organic F concentrations in the collected penguin bones (Table 1). About two-thirds of the F in their bones is in organic forms. The total concentration of F in penguin wing bone in relation to arthrosis is consistent with the sum of determined inorganic and organic F concentrations.

*Mineral and chemical structure analysis:* To characterize the organic F, X-ray diffraction, electronic energy spectrometry, and infrared spectrometry of penguin wing bones were performed with the results shown in Figures 1–3. As shown in Figure 1, the dominant mineral in penguin bone (including wing arthrosis PB-01, and wing bone PB-02) is carbonate-hydroxyapatite (OHAp); fluorated OHAp is not visible in the diagram. Figure 2a shows that the 1s electron of carbon has a signal at 290.5 eV, corresponding to a very high bond energy that suggests the existence of covalent C–F bonds. As shown in Figures 2b and 2c, nitrogen is mainly present in the amido form. Penguin bones have infrared absorption peaks (Figure 3) at 3510-3230, 2980, 1680, 1450, 1040, 877, 609, 577, and 374 $cm^{-1}$, characteristic of the acetamido and amino functional groups of chitin or its derivatives. These structure-bonding analyses indicate that most of the organic F seems to be present in the form of fluorinated chitin derivatives.

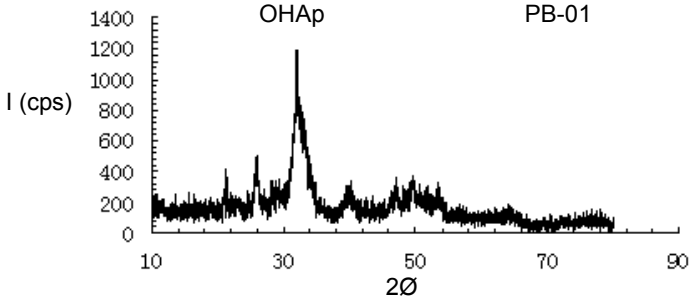
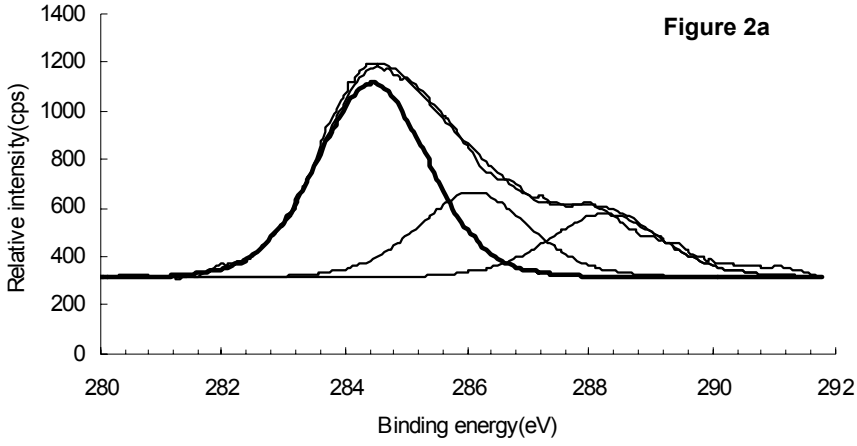
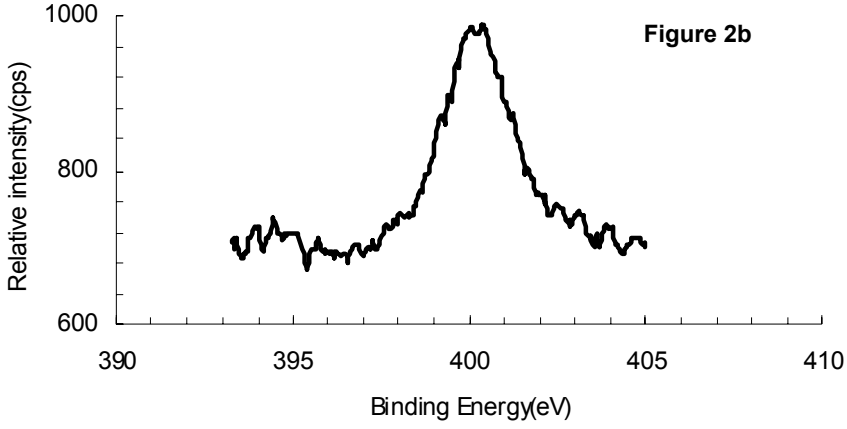
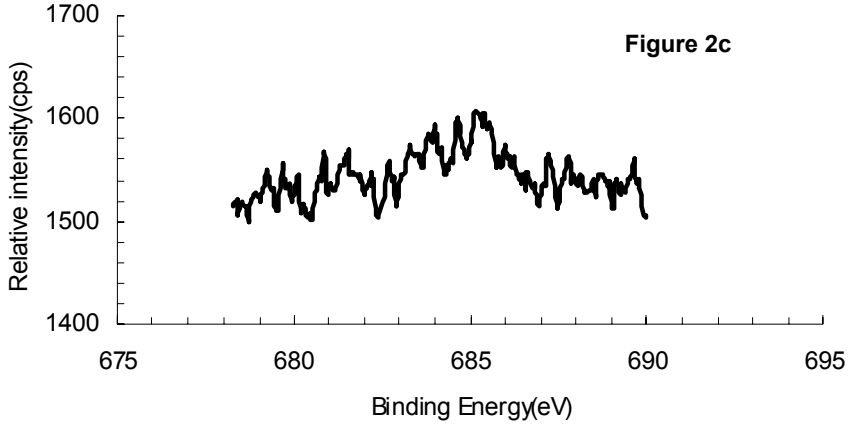



**Figure 1.** X-ray diffraction spectrogram for penguin wing arthrosis (PB-01) and wing (PB-02) bone powder.

Chitin (β-1,4-linked polymer of N-acetyl-D-glucosamine) is a key structural component of crustacean exoskeletons, and it is the most important carbohydrate in the diets of many marine carnivores. Rockhopper Penguins (*Eudyptes chrysocome*), Gentoo Penguins (*Pygoscelis papua*), and King Penguins (*Aptenodytes patagonicus*) fed on Antarctic krill (*Euphausia superba*) retain a substantial proportion (52.8±37.6%, 45.3±5.6%, and 84.8±11.7%, respectively) of ingested chitin.[14] *Euphausiids*, the main kind of krill in Adélie penguin's diet, has the highest overall fluoride concentrations among the Antarctic marine crustaceans examined, and the F in *Euphausiids* is concentrated in the chitin exoskeleton with levels up to 5477 μg/g.[11] In *Euphausiids* crust, half the total F is present in organic form.[10] Chitin and the F from krill are digested by penguins and retained in their bones.




**Figures 2a, 2b, and 2c.** Electronic energy spectrogram of C, N, F for penguin wing arthrosis (PB-01) bone powder.

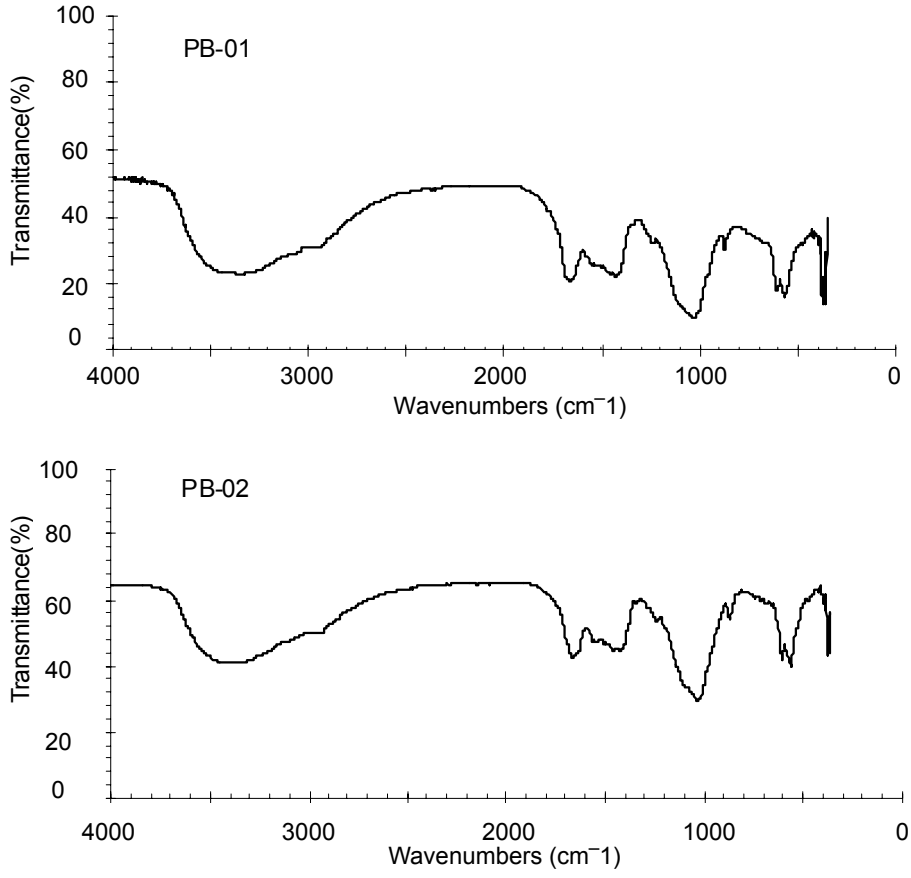



**Figure 3.** Infrared spectrogram for penguin wing arthrosis (PB-01) and wing bone (PB-02).

*Effects of chitin on F metabolism in experimental animal rat:* Figure 4 presents the bone mineral density (BMD) of the three groups of rats. The BMD value was significantly higher in the F-exposed group as compared with the control and the F-exposed + chitin groups. While there was no significant difference between the F-exposed + chitin group and the control group ($p>0.05$), this result demonstrated that chitin reduced the high BMD value caused by high-dose F.

Figure 5 shows the levels of inorganic F in the plasma. A significantly higher inorganic level of F in the plasma was found in the F-exposed group as compared with control and F-exposed + chitin groups. Although it was still higher than that of the control group ($p<0.05$), the toxic concentration of inorganic plasma F in the F-exposed + chitin group was significantly lower than in that of the F-exposed group ($p<0.05$), This result demonstrated that chitin reduced the F concentration in the plasma of F-exposed rats.

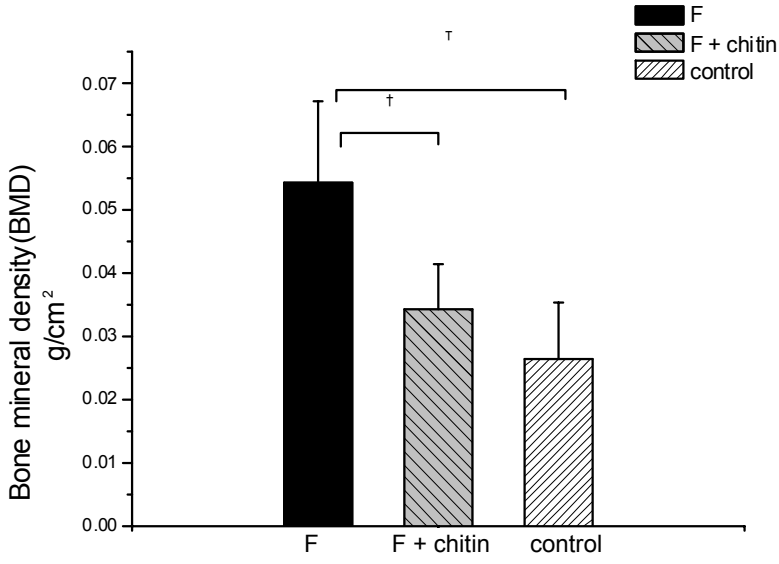



**Figure 4.** Bone mineral density (BMD) values in rat tibiae bone of control (n=9), F-exposed (n=7) and F-exposed + chitin (n=10) groups. (*p<0.05; †p<0.01)

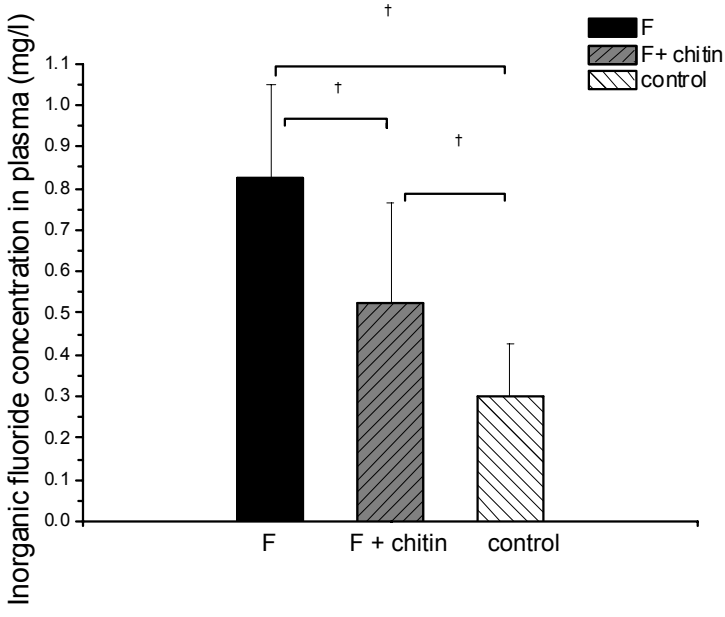



**Figure 5.** Inorganic fluoride levels in rat plasma of control, F-exposed and F-exposed + chitin groups. (*p<0.05; †p<0.01)

The total F concentration in the tibiae bone of the rats is shown in Figure 6. The F content of these bones was significantly higher in the F-exposed group than in the control and F-exposed + chitin groups (p<0.05). Although higher than that of

the controls, the concentration of F in the bones of in the F-exposed + chitin group was lower than in F-exposed group ($p<0.05$). This result indicates that chitin reduced the total F concentration in the bones of F-exposed rats.

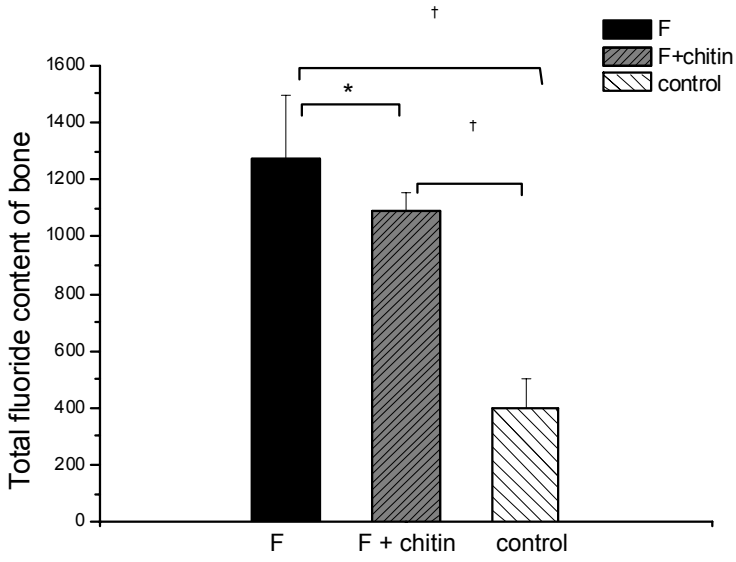



**Figure 6.** Total fluoride levels in rat tibiae bone of control, F-exposed and F-exposed + chitin groups. (*p<0.05; †p<0.01)

Tables 2 and 3 give the inorganic and organic F concentrations in the tibiae bone.

**Table 2.** Inorganic fluoride concentration (µg/g) in tibiae bones of experimental rats

| Group | Mean ± SD | p value |
|---|---|---|
| Control | 177.12 ± 55.53 | |
| F-exposed group | 390.23 ± 49.71 | *p<0.01 |
| F-exposed + chitin group | 311.61 ± 29.95 | *p<0.01; †p<0.05 |

*compared with controls; †compared with F-exposed group.

**Table 3.** Organic fluoride concentration (µg/g) in right tibiaes of experimental rats

| Group | Mean ± SD | p value |
|---|---|---|
| Control | 280.30 ± 161.41 | |
| F-exposed group | 852.39 ± 68.65 | *p<0.05 |
| F-exposed + chitin group | 755.67 ± 87.68 | *p<0.05; †p>0.05 |

*compared with controls; †compared with F-exposed group.

Compared with controls, the inorganic F concentration in the F-exposed group ($p<0.05$) was obviously higher, and it was significantly reduced to 311.61 ± 29.95 μg/g by chitin treatment ($p<0.05$). This result demonstrates that chitin can substantially reduce the inorganic F concentration and thus alleviate the damage caused by high inorganic F, even though there was no significant difference in the organic F content between the F-exposed and F-exposed + chitin groups ($p>0.05$).

*Possible reasons for penguins not having fluorosis:* The ideal bone mineral formula is calcium hydroxyapatite: $Ca_{10}(PO_4)_6(OH)_2$.[15] The primary cause of fluorosis is that inorganic F ion substitutes the hydroxyl group and affects the crystal texture of carbonate-hydroxyapatite, such as crystal size and BMD.[16]

The above analyses and animal experiments suggest that a number of factors act together and prevent fluorosis in penguin bones, in which two thirds of the F is present in an organic form that cannot substitute for the hydroxyl group in hydroxyapatite, or at least less effectively. Julshamn et al.[17] also reported that Atlantic salmon (*Salmo salar*) is highly tolerant of dietary F given as krill meal with a F concentration up to 350 μg/g and that the F concentration in muscle, whole body, and bone of Atlantic salmon is not affected by the dietary fluoride level over a short period of time.

Although from our study it appears that most of the F in penguin bones is present in organic form, the inorganic F level is about 3200 μg/g, which still too high for normal bone development. As seen from our laboratory animal experiment, chitin, having a high level in penguin bones, very likely plays a role more than simply forming an organic complex with F. Chitin is apparently also involved in biomineralization[18] and helps to promote bone formation and strengthen calcium phosphate cements.[19] For the control, F + chitin, and F-exposed groups, the proportion of inorganic F in total F was 38.7%, 31.4%, and 29.2%, respectively.

Various studies have focused on the effects of inorganic fluoride on bone mineralization, which, in essence, have been found to increase the size of the apatite crystals and promote abnormal bone formation. Although the F-exposed + chitin group in our animal experiment has a higher proportion of inorganic F in total F than in the F-exposed group, chitin deceased the BMD, and inorganic F in plasma. We thus deduce that chitin can help animals to resist the harm from high-dose F exposure. Second, it might inhibit F absorption and promote F, which can help alleviate intoxication from inorganic bone F. Compared with the F-exposed group, the F-exposed + chitin group had its total bone F content reduced by 21.23% and the inorganic F in plasma by 36.13%. Recent work has shown that about 75% of F absorption occurs from the small intestine and the rest from the stomach.[20] F is also effectively absorbed in the form of the liposoluble calcium monofluorophosphate (MFP) complex[21] excreted via the kidneys in relation to urinary pH, with a dynamic balance between absorption and excretion.[22] We therefore speculate that chitin can effectively decrease F absorption. However, further studies are required to investigate how chitin affects F metabolism.

Finally, we note that our findings may have potential applications for preventing or arresting fluorosis. To reverse fluorosis, chemical medicaments like selenium,[23] boron,[24]and the mineral of serpentine have been used to increase F excretion. However, the efficacy of these treatments is usually not permanent, since the patients usually have continued F intake afterward. The clinical addition of chitin (such as shrimp) to the diet could, however, be a promising method for prevention and reversal of skeletal fluorosis on long-term scale. Moreover, the beneficial effects would be better if F in chitin is first removed as by the method proposed by Tenuta.[25] Although it still needs further investigation, especially for the molecular mechanism of the action of chitin on F in bone cells,[26] this study offers the possibility of a feasible and inexpensive method to help alleviate the suffering of approximately 300 million people afflicted with skeletal fluorosis around the world, especially in China[27-30] and India.[2]

In conclusion, based on our animal experiments, we found that chitin, by absorption of inorganic F, might prevent abnormal mineralization of bone by decreasing excessive BMD and improving bone histomorphometry. Combined with the finding that nearly two-thirds of the total F in the penguin bones is organic F, we can better understand why penguins do not get skeletal fluorosis from a high-F intake from a F-rich krill diet.

**ACKNOWLEDGEMENT**

Financial support from the National Natural Science Foundation of China (No. 40476001 and 40231002) is greatly acknowledged.

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