

Letter

## Selenium increases expression of HSP70 and antioxidant enzymes to lessen oxidative damage in Fincoal-type fluorosis

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**ABSTRACT** — Fincoal type fluorosis has only been reported from China, but its pathogenesis is unclear. Many people believe that fluorosis is associated with oxidative stress. Oxidative stress can be reduced at higher selenium (Se) level. Heat shock protein (HSP70) is the most conserved and induced against different stressors. The aim of this study is to detect the expression of HSP70 in fluorosis patients and explore the role of Se in fluorosis protection. The subjects were divided into four groups: “High Se + F group” (n = 50), “High F group” (n = 50), “High Se group” (n = 20) and “Control group” (n = 46). Expression of HSP70 was evaluated by Western blotting and real-time PCR techniques. The concentration of fluoride, content of Se in hair, activity of antioxidant enzymes (GSH-Px, SOD, CAT) and content of malondialdehyde (MDA) were determined. The relative amount of HSP70 gene transcription was significantly higher in “High Se + F group” than the other groups. The same results were found for expression of HSP70 protein to  $\beta$ -actin ratio. There was a significant difference between “High Se + F group” and “High F group” regarding MDA content and glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT) activity. These results suggest that oxidative stress plays an important role in the pathogenesis of the Fincoal type fluorosis and it can be reduced at higher Se level.

**Key words:** Fincoal type fluorosis, HSP70, Real-time PCR, Oxidative stress, Enzyme inhibition, Fluoride

### INTRODUCTION

Fluoride is naturally present in varying amounts in air, water and food and its excessive accumulation in the body can exert toxic effects (Spittle, 1994). Endemic fluorosis that seriously impairs human health is prevalent in the world. The latest estimates suggest that about 200 million people, from among 25 nations the world over are affected by the serious problem of fluorosis (Ayoob and Gupta, 2006).

Endemic fluorosis is prevalent in China (Zhang, 2008) because of high fluoride water (Luo *et al.*, 2008), brick tea (Cao *et al.*, 2004) and indoor combustion of high-F coal (Ando *et al.*, 2001). Eight counties of Ankang City are acutely affected areas in Shaanxi province with Fincoal-type fluorosis (Xu *et al.*, 2008). Among these counties, Ziyang County is famous for its selenium-rich tea,

and has higher environmental selenium (Se) level, with Haoping Town having the highest level (Fang and Wu, 2004). We selected fluorosis Patients from Haoping Town as “High Se + F group”.

Patients from Hanbin district of Ankang City were selected as “High F group” because its environment Se level is the lowest among the eight counties and is still within normal range (Yang, 1989). We also selected healthy volunteers from Haoping Town as “High Se group” and healthy volunteers living in Xi'an as “Control group” which is the capital of Shaanxi province and has normal Se level in its environment.

Investigators have explored the mechanism of fluorosis, consequently, collected numerous biological evidences, including oxidative stress (Izquierdo-Vega *et al.*, 2008), apoptosis (Zhang *et al.*, 2007), and over-load of intracellular calcium (Xu *et al.*, 2007). Among these the-

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ories, the “Oxidative stress” is the only explanation that can explain the pathological lesion of multiple organ and multisystem fluorosis (Guan *et al.*, 2007).

Oxidative stress in biological systems results from an imbalance between cellular reactive oxygen species (ROS) and capacity of antioxidants. The antioxidants not only play a key role as ROS scavenger, but also regulate the redox state of the cells (Matés *et al.*, 1999). In oxidative stress, antioxidants such as GSH, glutathione peroxidase (GSH-Px) and SOD are expended, the ability of antioxidation reduces, and the product of lipid peroxidation, i.e. malondialdehyde (MDA) accumulates (Ozgoçmen *et al.*, 2007). The involvement of antioxidants in people responding to fluoride toxicity is unclear because it does not belong to the group of transition metals that may induce oxidative stress. The mechanisms by which fluoride acts at the cellular level and by which people may defend themselves against this pollutant are not fully understood yet.

Among the families of stress proteins, heat shock protein (HSP70) is the most conserved and one of the first HSPs to be induced against different stressors. Conserved nature of the stress response across species allows the application of HSPs based assays over a wide range of organisms and in this regard HSP70 expression has been proposed as a sensitive indicator of adverse biological effects (Varó *et al.*, 2002; Mukhopadhyay *et al.*, 2003).

The present study was aimed to detect the correlation between HSP70 expression and antioxidant enzymes in fluorosis, beneficial role of Se in fluorosis to lessen oxidative damage and the relationship of HSP70 expression with Se.

## MATERIALS AND METHODS

### Patients and groups

According to the fluoride concentration of the coal and dental fluorosis status, which was assessed by Dean’s index, a total of 100 patients (age ranging from 28 to 68) were selected including; fifty patients from gaige, ping-

chuan and shuangxing village of Haoping Town as “High Se + F group”, twenty healthy people from the hejiatai, xianghe village of Haoping town as the “High Se group”, Fifty patients from the shuangqin and shuanglong villages of the Hanbin District as “High F group” and forty-six healthy volunteers living in Xi’an as “Control group”. No statistical significant difference was found in gender and age among all the groups (Table 1). The patients were informed about the purpose of research and their consent was taken before enrolment. The local Ethics Committee approved the study protocol in accordance with institutional guidelines.

### Sample collection

Approximately 6 ml of blood and 10 ml instant urine was collected from each person (fast, morning). EDTA was added in 3 ml of blood for anticoagulation, and Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (2,500 rpm/min for 15 min). Serum was separated from remaining 3 ml blood by centrifugation at 3,000 rpm/min for 15 min. Serum and urine were kept at  $-20^{\circ}\text{C}$  until used for further analysis.

### Materials

Trizol Reagent was purchased from Invitrogen life technologies (Carlsbad, CA, USA). The rabbit anti-human HSP70 antibody was from Cell Signaling Technology Inc. (Beverly, MA, USA). The rabbit anti-human  $\beta$ -actin antibody was purchased from Lab Vision Corporation (Fremont, CA, USA). The horseradish peroxidase-conjugated anti-rabbit secondary antibodies were bought from Santa Cruz Biotech, Inc. (Santa Cruz, CA, USA). polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). SYBR PrimeScript™ RT-PCR Kit was obtained from Takara (Takara Shuzo, Shiga, Japan). RevertAid First Strand cDNA Synthesis Kit was purchased from Fermentas (Fermentas, MD, USA). The AEC staining kit was from Beijing Bio-Lab Materials Institute (Beijing, China). All primers were synthesized and purified by Beijing Sunbi-

**Table 1.** Gender and age in each group

Group	Gender (male/female)	Age
High Se + F group	21/29	46.73 $\pm$ 0.97
High F group	22/28	50.48 $\pm$ 3.10
High Se group	9/11	45.95 $\pm$ 1.67
Control group	20/26	49.17 $\pm$ 2.14

Note. Data are expressed as mean  $\pm$  standard error mean (S.E.M).  $P < 0.05$  compared with the control group. Data were analyzed with one-way analysis of variance (ANOVA).

## Fincoal type fluorosis and HSP70

otech Corp. (Beijing, China). Sodium fluoride (NaF) was obtained from Shanghai Chemical Reagent Corp. (Shanghai, China). The GSH-Px, SOD and CAT test kits were purchased from Jiancheng Bioengineering Ltd. (Nanjing, China). All other reagents were of analytical grade and all solutions were prepared using thrice-distilled water. All other reagents were obtained from commercial sources.

### Detections of fluoride concentration

Fluoride concentration was determined from samples previously stored at  $-20^{\circ}\text{C}$  by the method of Anon (1989), briefly, equal volumes of total ionic strength adjustment buffer (TISAB) solution (TISAB: 58 g NaCl + 4 g trisodium citrate + 57 ml glacial acetic acid, pH = 5.0) and serum or urine were taken and fluoride concentration was detected by using a fluoride ion-specific electrode connected to a pH Meter (Model PHS-2) and coupled to a standard calomel electrode (Model 232) as reference. The accuracy and precision of the method employed was checked by the analysis of a standard reference material, consisting of a standard with high and low levels of fluoride (National Institute of Standards and Technology).

### Se levels

Approximately 1 g of hair were collected from the nape of the neck from each subject and Se level was detected by AFS-930, Atomic Fluorescence Spectrophotometer (Titan, China) following the method of Wietecha *et al.* (2005).

### Detections of lipid peroxidation and the activities of antioxidant enzymes

The content of MDA, the activities of GSH-Px, SOD and CAT were measured from serum using commercial kits. The results were expressed as nmol/ml for MDA, U/ml for CAT,  $\mu\text{mol/l}$  for GSH-Px, U/ml for SOD, respectively.

### Western blot analysis

Total protein was extracted from PBMCs following the method of Hummon *et al.* (2007). The protein content was quantified by BCA reagent (Pierce Chemical Co., Rockford, IL, USA). The protein was adjusted to coincident concentration by the BSA standard curve and diluted with 1% SDS. Total protein extracted from PBMCs was resolved on 10% SDS-PAGE gel and transferred to PVDF membranes. Each sample of 30  $\mu\text{l}$  contains 20  $\mu\text{g}$  total protein. The PVDF membranes were dried at  $37^{\circ}\text{C}$  for 1 hr and blocked in 2.5% non-fat milk in Tris-buffered saline (TBS) with 0.01% Tween 20 at  $25^{\circ}\text{C}$  for 1 hr. The PVDF membranes were incubated overnight with prima-

ry antibodies at  $4^{\circ}\text{C}$ , washed, and incubated with second antibodies at  $37^{\circ}\text{C}$  for 1 hr. Immunoreactive proteins were visualized using the AEC staining kit. Densitometry was performed using Work-Lab software (UVP, Upland, CA, USA). The data was recorded as the ratio of sample to  $\beta$ -actin. All of the immunoblots were performed at least three times.

### Gene expression analysis by real-time quantitative RT-PCR assays

Total RNA was isolated from PBMCs following the method of Hummon *et al.* (2007). RNA was purified using RNeasy Mini Kit according to the supplier's protocol, quantified by optical density at 260 and 280 nm and stored at  $-80^{\circ}\text{C}$ . cDNA was synthesized from 3  $\mu\text{g}$  total cellular RNA with a 1st strand cDNA Synthesis Kit for RT-PCR. Quantitative real-time PCR was performed with the iQ<sup>TM</sup>5 Real Time PCR Detection Systems in 8 even-tubes and using SYBR PrimeScript<sup>TM</sup> RT-PCR Kit in 20  $\mu\text{l}$  reaction mixtures (SYBR 10  $\mu\text{l}$ , cDNA 2  $\mu\text{l}$ , forward primer 400 nM, reverse primer 0.5  $\mu\text{l}$ , RNase Free distilled H<sub>2</sub>O 7  $\mu\text{l}$ ) by using primers for human HSP70 mRNA (forward 5'-AGAAGGACATCAGCCAGAAC-3' and reverse 5'-GAACAGGTCCGAGCACAG-3') and  $\beta$ -actin (forward 5'-GAACGGTGAAGGTGACAGCAG-3' and reverse 5'-GTGGACTTGGGAGAGGACTGG-3'). The reaction mixtures were initially heated at  $95^{\circ}\text{C}$  for 10 sec followed by 40 cycles of 5 sec at  $95^{\circ}\text{C}$ , 15 sec at  $61^{\circ}\text{C}$ , and 10 sec at  $72^{\circ}\text{C}$ . The PCR products were resolved on a 2% agarose gel and densitometry was performed to quantify the amounts of HSP70 mRNA relative to the levels of  $\beta$ -actin (Data not show).

The sizes of PCR products were 182 bp and 200 bp for HSP70 and  $\beta$ -actin, respectively. The PCR product was evaluated by melting curve analysis using the iQ<sup>TM</sup>5 software (version 2.0). HSP70 mRNA data was normalized by  $C_T$  of  $\beta$ -actin, which is a non-regulated housekeeping gene. The cycle counts ( $C_T$ ) represents the relative abundance of a transcript.

Real-time quantitative PCR data was processed applying relative quantification using the  $\Delta\Delta C_T$ -method ( $2^{-\Delta\Delta C_T}$ ) (Livaka and Schmittgen, 2001).

### Statistical analysis

The data was expressed as mean  $\pm$  S.E.M. Differences between groups were evaluated by one-way ANOVA with subsequent Dunnett's t-test using SPSS13.0 for windows (Version 13.0, SPSS Inc., Chicago IL, USA). A difference at  $P < 0.05$  was considered statistically significant.

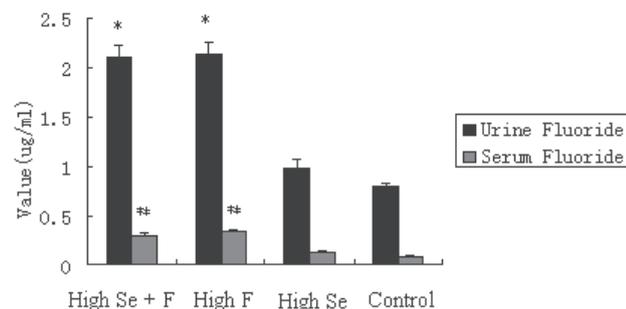
## RESULTS

### The fluoride contents in urine and serum

Fluoride content in the serum and urine was significantly higher in people with fluorosis in residential areas with high coal fluoride as compared to “Control group”. The fluoride content of serum and urine in “High Se group” was higher than that of in “Control group”, but has no statistical significance.

### The selenium contents in hair

Selenium content was significantly higher in “High Se + F group” and “High Se group” as compared to “High F group” and “Control group”. There was a slight increase in hair Se content in “High F” group as compared to “Control group”, which is not statistically important (Fig. 2). Results showed that none of subjects in this study is Se deficient according to marginal thresholds established in China (Yang, 1989), which implies that participants must have consumed relatively Se-rich foods.



**Fig. 1.** Concentration of fluoride in serum and urine. Concentration of fluoride in serum and urine was calculated from a standard curve, which was made from a reference material. \* Statistically significant ( $p < 0.05$ ).

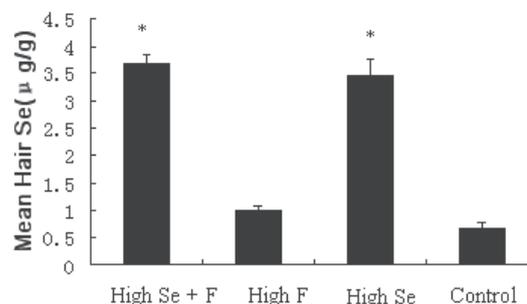
### The activity of antioxidant enzymes and the content of MDA

SOD, CAT and GSH-Px activity of “High F group” was lower as compared with “Control group”, with SOD and GSH-Px having statistical significance. CAT activity was significantly higher while GSH-Px activity lower in “High Se + F group” as compared with “Control group”. SOD and CAT activity was significantly higher while GSH-Px lower in “High Se group” as compared to “Control group”. All the enzymes have significantly higher activities in “High Se + F group” as compared to “High F group” (Table 2).

MDA content was significantly higher in “High F group” as compared with other groups, and there was significant decrease in the MDA content of “High Se group” and “High Se + F group” as compared to “High F group” (Table 2).

### HSP70 and $\beta$ -actin protein expression by western blotting.

HSP70/ $\beta$ -actin ratio was significantly higher in “High Se group” and “High Se + F group” as compared with the



**Fig. 2.** Content of Se in hair. According to the reference standard of selenium deficiency, the content of selenium lower than 0.66  $\mu\text{g/g}$  was described as selenium deficiency. \* Statistically significant ( $p < 0.05$ ).

**Table 2.** Activities of SOD, CAT, GSH-Px and content of MDA in each group

Group	SOD (U/ml)	CAT (U/ml)	GSH-Px ( $\mu\text{mol/l}$ )	MDA (nmol/ml)
High Se + F group	82.21 $\pm$ 3.93 <sup>#</sup>	21.63 $\pm$ 1.37 <sup>**#</sup>	313.28 $\pm$ 10.22 <sup>**#</sup>	5.07 $\pm$ 0.28 <sup>**#</sup>
High F group	55.56 $\pm$ 4.93 <sup>**</sup>	3.35 $\pm$ 0.16	194.65 $\pm$ 10.76 <sup>**</sup>	24.09 $\pm$ 1.60 <sup>**</sup>
High Se group	115.05 $\pm$ 6.19 <sup>**#</sup>	15.90 $\pm$ 2.26 <sup>**#</sup>	243.41 $\pm$ 33.87 <sup>**</sup>	5.74 $\pm$ 0.36 <sup>**#</sup>
Control group	86.65 $\pm$ 9.20 <sup>#</sup>	6.45 $\pm$ 0.38	445.58 $\pm$ 8.51 <sup>#</sup>	11.01 $\pm$ 1.08 <sup>#</sup>

Note. Data are expressed as mean  $\pm$  S.E.M.

<sup>\*\*</sup> $P < 0.01$  compared with the control group.

<sup>#</sup> $p < 0.01$  as compared with “High F group”. Data were analyzed with one-way ANOVA.

“Control group”. However, there was no significant difference in HSP70/ $\beta$ -actin ratio between “High F group” and “Control group” (Fig. 3).

### Real-time quantitative PCR analysis of PBMCs

HSP70 mRNA level (relative amount) in “High Se + F group” was significantly higher as compared to “High F group” and “High Se group”. No significant difference was found when mRNA level of “High Se group” and “High F group” were compared (Fig. 4).

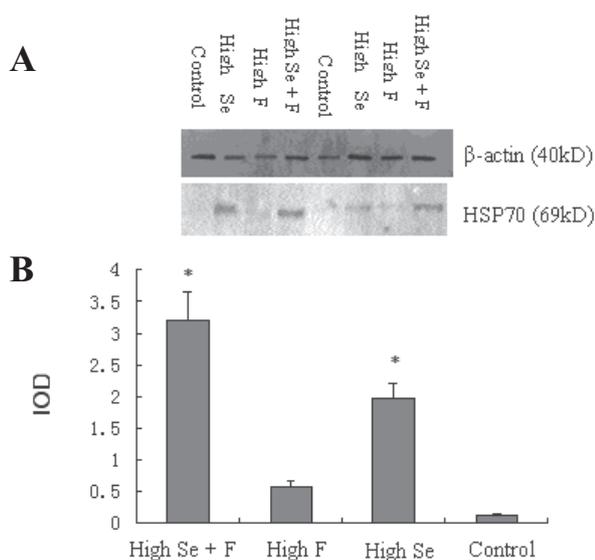
### The correlation between HSP70 gene transcription and protein expression

A positive correlation was found between HSP70 gene and protein expression ( $r = 0.568$ ,  $P < 0.001$ ). The expression of HSP70 gene was significantly higher in “High Se + F group” as compared with “High F group” (Fig. 4). Similar results were observed when these two groups were compared for protein expression (Fig. 3).

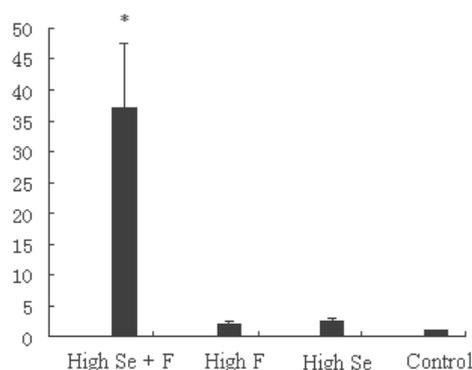
## DISCUSSION

Different authors have postulated different pathogenic mechanisms of fluorosis. The “Oxidative stress” is the key point of fluorosis at present (Guan *et al.*, 2007). Oxidative stress is defined as a disturbance in the balance between the production of free radicals and antioxidant defense. The hypothesis regarding that oxidative stress may be involved in the pathogenesis of chronic fluorosis has been emphasized, based on results from many investigations on patients, animal models with chronic fluorosis, and cultural cells treated with high-concentration of fluoride. These findings have shown high level of MDA from lipid peroxidation (Shivashankara *et al.*, 2002), and reduced activities of antioxidant enzymes (Chlubek *et al.*, 2003).

Many organisms need Se in order to maintain antioxidant (Stadtman, 1990). Se has been found and accepted as an essential trace element for living organisms, including humans, because it forms the active center of GSH-



**Fig. 3.** Selenium increased HSP70 protein expression. The total protein extract of PBMC was subjected to PAGE and sequential Western blot analysis with HSP70 specific antibody against HSP70 followed by incubation with anti-rabbit secondary antibody and revelation by AEC staining. Integral optical density (IOD) was calculated by Gel Imaging System automatically. The positions of HSP70 and  $\beta$ -actin were shown (A). The mean densitometric values of each sample protein divided by  $\beta$ -actin from three independent experiments were depicted as bar graphs (B). Values are presented as the mean  $\pm$  S.E.M. \* $P < 0.05$  vs control.



**Fig. 4.** Effects of Se on HSP70 mRNA expression by real-time PCR. To examine the expression of HSP70 mRNA real-time quantitative RT-PCR was carried out.  $\beta$ -actin was used as quantitative relative internal parameters to normalized HSP70 expression level. Relative amount in sample =  $2^{-\Delta\Delta C_T}$ .

Px that plays a role in relieving severe oxidative stress (Rotruck *et al.*, 1973). The positive effect of Se has been demonstrated as a decrease in lipid peroxidation and as an increase in the activity of anti-oxidative enzymes.

We observed a significant increase in MDA concentration in serum in "High F group", suggesting oxidative damage had occurred. Our results indicate that activities of GSH-Px, SOD and CAT can be decreased in chronic fluoride exposure, whereas Se can inhibit the effect of fluoride on antioxidant enzyme activities and increase the activities. Selenium is part of enzyme GSH-Px that catalyzes the oxidation of glutathione in the presence of hydrogen peroxide to yield oxidized glutathione and water. (Bounous and Molson, 2003). GSH-Px can protect the cells against oxidative damage. Se deficiency is accompanied by a decrease in GSH-Px activity (Wu *et al.*, 2003). So when chronic fluoride exposure occurs, the loss of GSH-Px activity is predominant.

We detected selenium from hair as it reflects long term intake of trace elements (Slotnick and Nriagu, 2006). HSPs are a family of stress-induced proteins exhibiting well described functions in cytoprotection preserving cell viability. Of all of the HSP families that have been studied, it is the HSP70 family that has been most widely used as a biomarker due to its rapid and significant increase during cellular stress (Ryan and Hightower, 1996). The fundamental role of HSP70 is to repair protein damage that can occur as a consequence of stress (Gething and Sambrook, 1992) and therefore this family of proteins plays a key function in cytoprotection. In response to environmental stresses, the gene coding for the inducible 69kDa HSP70 is rapidly activated.

Although reinforcing endogenous self-preservation mechanisms are considered as a promising strategy for advanced organ protection, the effect of HSP70 on chronic fluorosis damage has not been reported. Compared to control group, the expression of HSP70 protein was higher in "High Se group" ( $p < 0.001$ ). We studied the expression of HSP70 protein in our experiment because it was higher in "High F group" as compared to "Control group" and we found a remarkable increase in the level of HSP70 proteins in "High Se + F group" as compared to "High F group". A higher level of HSP70 mRNA on "High Se + F group" as compared to other groups demonstrates that high Se level significantly increases the expression of HSP70 in PBMCs and attenuates the oxidative stress caused by chronic fluorosis. These findings suggest a possible role of HSP70 as a mediator in inducing tolerance against chronic fluorosis.

In addition the present data provide additional indications that Se can up-regulate HSP70 mRNA (37 fold),

induce the high-expression of HSP70 protein (15 to 24-fold) and reduce the damage induced by fluoride intoxication in human PBMCs. The decrease of HSP70 mRNA and the quantity of HSP70 protein expression may result from oxidative damage, which would lead to a total loss of membrane fluidity and integrity (Horváth *et al.*, 2008). This would reduce the activities of GSH-Px, SOD and CAT enzyme available to lessen the oxidative damage, so that the content of MDA would increase.

Similarly, Se in our study also increased the basal steady-state HSP70 protein level in "High Se group", suggesting that Se exerts a mild stress on cell though the HSP70 mRNA level was not increased evidently. Moreover, PBMC from patients with high Se level had arguing for evidently improved ability to cope with stress. Since blood samples were obtained and frozen under controlled conditions at different times by the same personnel, the effect observed was not due to differential sample preparation.

We conclude that fincoal type fluorosis is linked with oxidative stress. Se is an inducer of HSP70 and has a major importance in stress response of human PBMCs in the fluorosis. Since the stress response is a conserved and universal phenomenon, and the effect of Se *in vivo* is fairly general, these findings may have wider implications in other body tissues as a possible anti-fluorosis mechanism. However, whether this is a proof of principle or a special exception will be a subject of future studies.

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