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# Examination of the Roles of Selenium in the Kaschin-Beck Disease

## Cartilage Cell Test and Model Studies

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## ABSTRACT

The Kaschin-Beck Disease, an endemic disease in China, occurs in low-selenium areas. Using human embrionic cartilage cell as a system, the effect of selenite and another etiological factors, such as, organic matters in water, and grain from disease regions, were studied. It was shown that Se(IV), as well as superoxide dismutase, could prevent the cells from damage by organic matters, and increase the activity of GSHpx and decrease the production of lipid peroxide. A model test of adrenalin autooxidation was carried out, and it was found that the oxy-radical can be elminated by Se(IV). Thus, it was assumed, that selenium was a protective factor and free radical scavenger for Kaschin-Beck Disease.

**Index Entries:** Selenium; Kaschin-Beck Disease; cartilage cell; fulvic acid; toxin of Fusarium; oxygen free radical.

## INTRODUCTION

The Kaschin-Beck disease (K.B.D.) is an endemic disease in China. The basic pathological change of this disease, occuring mainly in children, is the multiple generation and necrosis of articular cartilage and

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growth plate that can result in permanent disabilities (*1*). Surveys and experiments in China, Japan, and Soviet Union have been carried out in the last three decades for studying the possible etiology of K.B.D., but agreement has yet to be reached. Based on the environmental characteristics and practical preventing effects, it was found that K.B.D. always occurred in low-selenium areas (*2*), but selenium deficiency alone is not sufficient to be the cause of this disease. The etiology remains obscure, but has been found to be associated with the chemical matters in natural water, and possibly the staple food (*3*). Humic acid (HA), the main component of the organic matter in natural water (*4*), and the toxin of Fusarium Oxysporum (FM) in grain (*5*), were suspected to be the other causes. Recently it was assumed that K.B.D. is caused by a combination of local environmental factors, including the selenium deficiency. The relationship between selenium and HA in water (*6*), as well as selenium and toxin of FM in grain, have been emphasized in our laboratory (*7*). From mice experiments, it was found that selenium could inhibit greatly the toxicity of fulvic acid (FA), soluble fraction of HA extracted from drinking water in disease regions, or of the FM toxin isolated from contaminated grain.

The pathological process of K.B.D. has already been known. The initial lesion is selective coagulative necrosis of the hypertrophic cartilage, cells at the base of articular and growth plate cartilage, and cartilage cells were selectively subjected to damage by a toxic agent or the deficiency of a required metabolite (*1,2*). In order to find out whether Se(IV) could also inhibit the toxicity of these organic matter in pathological process of K.B.D., the human embrionic cartilage cells was used as a system to study the effects of selenite and organic matters from water and contaminated grain. It was shown that selenite could prevent the cells from the damage by FA and FM. Similar phenomenon was observed as superoxide dismutase (SOD) was added to the cultural medium. Furthermore, the production of lipid peroxide in the cell system was measured, and the presence of free radical was identified, and Se could reduce its production. To study the mechanism of Se as a protective factor, finally, a model test by adrenalin autooxidation was carried out to generate superoxide, and it was found that Se(IV) could eliminate this oxyradical. From cartilage cell and model tests, it was proposed that pathogenic factor could be the radicals generated from either FA in water, or mycotoxin in grain, or in both, and selenium, as a protective factor and free radical scavenger, could decrease the damaging effects.

## MATERIALS AND METHODS

Human embryonic cartilage cell (HEC) culture: The culture system used in the study has been previously described by Wang (*8*). Simm's solution was used as the buffer solution, and nutrient mixture F-12 (HAM) (GIBCO) as the cultural medium. Cells were dissociated with

Table 1
Composition of the Tested Medium

| Group | Reagent and Concentration | |
|---|---|---|
| 1 | N | |
| 2 | FA | 50 ppm |
| 3 | FM | 150 ppm |
| 4 | Se(IV) | 0.1 ppm |
| 5 | SOD | 1μg/mL |
| 6 | Se(IV) | 0.1 ppm |
| | FA | 50 ppm |
| 7 | SOD | 1μg/mL |
| | FA | 50 ppm |
| 8 | Se(IV) | 0.1 ppm |
| | FM | 150 ppm |
| 9 | SOD | 1μg/mL |
| | FM | 150 ppm |

trypsin and grown on cultural medium or tested medium. Cells were incubated over 2 d at 37°C, and the cultural solution was replaced at 48 h intervals. The HEC was finally distributed at random into nine groups, one of these was provided even further with the standard media as described above, whereas the others were supplied with modified medium, shown in Table 1. Determination of fluorescent lipid peroxidation products in HEC system (*9*): Sampled cells were collected by centrifugation at 3000*g* for 5 min, medium replaced for washing by three of 0.2*M* sodium phosphate, pH 7.0, digested with 0.02% EDTA, 37° for 20 min, then were homogenized in 1mL sodium phosphate buffer solution at 5000 rpm, and separated by blowing. The homogenate was extracted by 6mL 1:2 methanol–chloroform solution in a 45°C water bath for 5 min. Water (6mL) was added for washing the organic phase in order to remove the flavins compounds and centrifugalized at 3000*g* for 2 min. The mixture was transferred to centrifuge tube, shaken, centrifugalized for 5 min, and removed the aquatic phase. A 1 mL aliquot of the organic layer was mixed with 1mL methanol in a quartz cuvette, and the fluorescence spectra were recorded by spectrofluorophotometer (RF-520 Schmadzu).

Activity of glutathione peroxidase (GSHpx) of HEC system assay (*10*): A cell suspension was diluted to 100 million $10^8$ cells/mL by 0.2*M* saline phosphate buffer, pH 7.0, and was homogenized at room temperature. In a 5.0 mL tube, 0.4 mL of 1.0 mM GSH added, 0.4 mL of cell homogenate, warmed on 37°C water both for 5 min, after a 5 min preincubation, 4.0 mL metaphosphoric acid precipitating solution was added, stirred, and centrifugalized at 3000 *g* for 10 min. The content of GSH in the solution was determined by mixing 2.0 mL of clear supernatant solution with 2.5 mL 0.32*M* $Na_2HPO_4$, 0.5 mL DTNB reagent. $A_{422}$ was recorded within five minutes after mixing ($OD_{sample}$). A blank (with

water substituted for cell suspension) was carried through the incubation simultaneously with the sample ($OD_{non\text{-}enx}$), since nonenzymatic GSH oxidation by $H_2O_2$ occurred during incubation. Absorbance of standard of GSH ($OD_{GSH}$) was determined by using 2.0 mL of 40 μ*M* GSH instead of 2.0 mL supernatant.

Activity of GSHpx was assayed as follows:

$$GSHpx\ (u) = \{OD_{non\text{-}eng} - OD_{sample}\}C_{GSH} / OD_{GSH} \times 5$$

Autooxidation curves of adrenalin: In an alkaline condition, adrenalin can produce superoxide anion $O_2^-$ through a chain reaction induced by trace metal ions. In a adrenalin solution added tested solution. Autooxidation curves were recorded within 2 min after mixing by UV-200 spectrophometer (Shimadzu).

Fulvic acid(FA): Extracted from Yongshou county disease region, and purified as described (*6*).

Toxin of Fusarium Oxysporum(FM): Fusarium Oxysporum NF 032 was provided by Institute of Microbiology, Academia Sinica, and isolated from corn Hei Longjiang disease region. The toxin was extracted as described (*7*), only using water instead of bean oil. The filtrate was distilled in vacuum for a certain volume ready for use.

Superoxide dismutase (SOD): SOD was provided by Suzhou College of Medical Sciences. Activity of SOD was 150 u/ng.

Sodium selenite and all reagents used were A.R. grade.

## RESULTS AND DISCUSSION

The morphology of HEC was studied. The cells treated with FA and FM were different from the control cells. The cells of group 1 (Table 1) grew well, higher density, body opulent, nucleus clear, and no obvious polygonal protuberance. The main changes of groups 2 and 3 were cell swelling and accumulation of a large quantity of degenerative products. Cells of groups 2 and 3 were slender or body broken, obviously polygonal protuberance, poor density with blank areas, and multivesicular bodies and lipid bodies appeared. Karyoclasis and ghost cells were also seen. The shapes of nuclei became irregular, as compared to the control group. All these were caused by the cell membrane defects induced by FA and FM. As Se or SOD was added into the culture medium (groups 4,5), and either Se (groups 6,8) or SOD (groups 7,9) was added into FA or FM culture medium, the cells appeard to be normal and similar to the control group.

These results indicated that both FA and FM could damage the cell, but selenite could prevent the cells from the damage. These results obtained from cell tests correspond with our results in previous animal studies, in which Se could inhibit the toxicities of both FA (6) and FM (7) were shown.

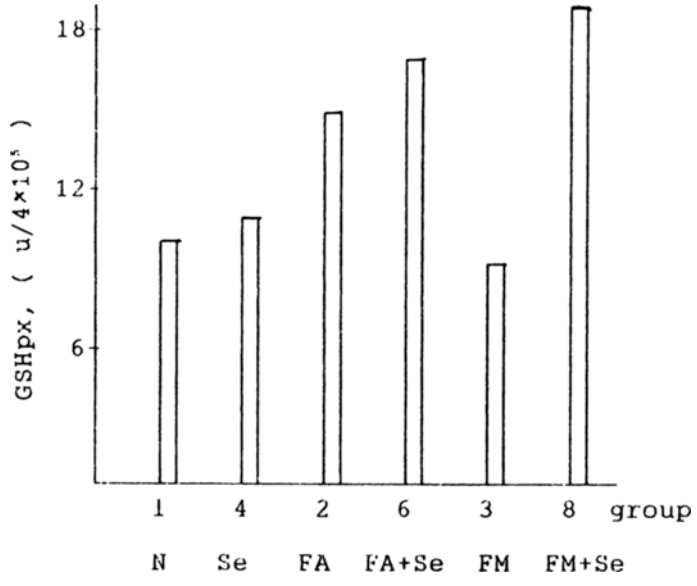


Fig. 1. Activities of GSHpx in HEC.

The activities of GSHpx of the cell systems are shown in Fig. 1. Se(IV) increased the GSHpx levels. GSHpx, the important selenoenzyme catalyzed the reduction of organic hydroperoxides and hydrogen peroxides, and it is an essential component of the cellular antioxidant system. It is interesting to note that similar function was observed as SOD was added into the culture medium, including FA and FM. As SOD can catalyze the dismutation reaction of superoxide anion $O_2^{\cdot}$, it may be assumed that the damage action of both FA and FM involved the production of active oxygen free radical. To confirm this assumption, the fluorescent lipid peroxide content in HEC were assayed. The results are summarized in Fig. 2, that indicates the enhancement of the degree of lipid peroxidation in the FA and FM (groups 2,3). However, Se and SOD reduced lipid peroxidation enhanced by both FA and FM (groups 6–9). It is likely that the damage of FA and FM on HEC were actually caused by oxyradical formed in the system, and the function of Se as well as SOD in protecting HEC from damage was related to their inhibiting action on lipid peroxidation.

The ESR spectra of FA and FM were recorded by the electron spin resonance spectroscopy (*11,12*), and the typical ESR spectra with g factor values between 2.003–2.004, were shown, and were identified as to be caused by semiquinone (QH). It is known that under aerobic conditions,

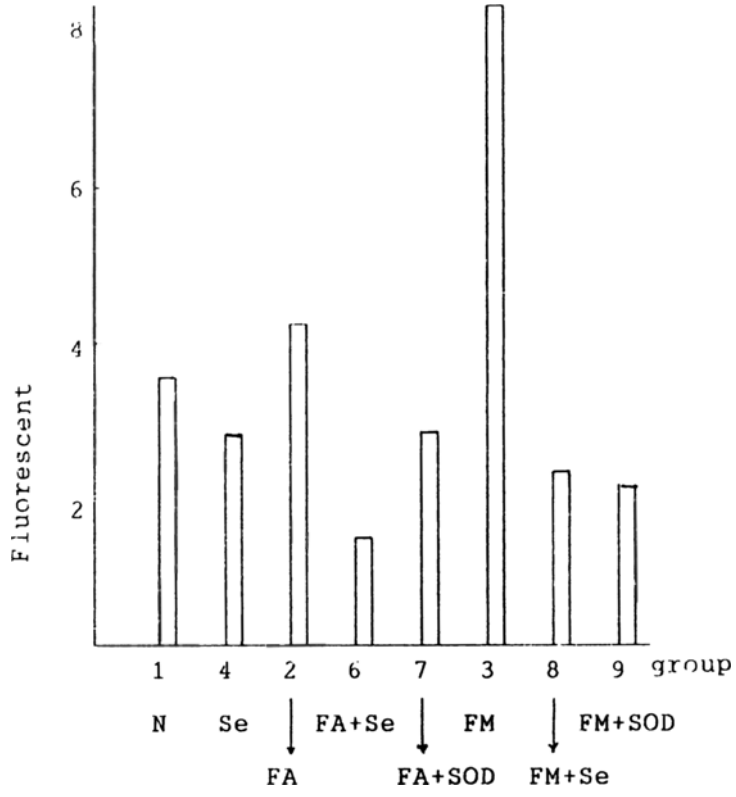


Fig. 2. Variations of fluorescent lipid peroxidation products in HEC.

a semiquinone radical autooxidizes and forms the superoxide anion radical, that could initiate lipid peroxidation and cause deleterious effects. As oxyradicals may depolymerize chondroitin sulfate and decrease its viscosity (*14*), this provides a clue to the role of free radical generated by FA and FM in the causes of KBD. Selenium could increase the activity of GSHpx and decrease the lipid peroxidation in HEC system, thus, it was assumed to prevent the formation of oxyradical as a scavenger.

To reveal the mechanism of selenium as a protective factor, and the functions of both FA and FM, a model test was carried out by adrenalin (AL). The adrenalin system that could generate superoxide anion from its autooxidation process (*13*) in solution can be expressed as a function of optical density at 480nm. Subgroup expermiments were arranged as follows: (A) AL alone; (B) AL+FA; (C) AL+FM (D) AL+FA+Se; (E) AL+FA+SOD; (F) AL+FM+Se; (G) AL+FM+SOD. Experiment results indicated (Figs. 3–5) that there is an increase in $O_2$ in groups B and

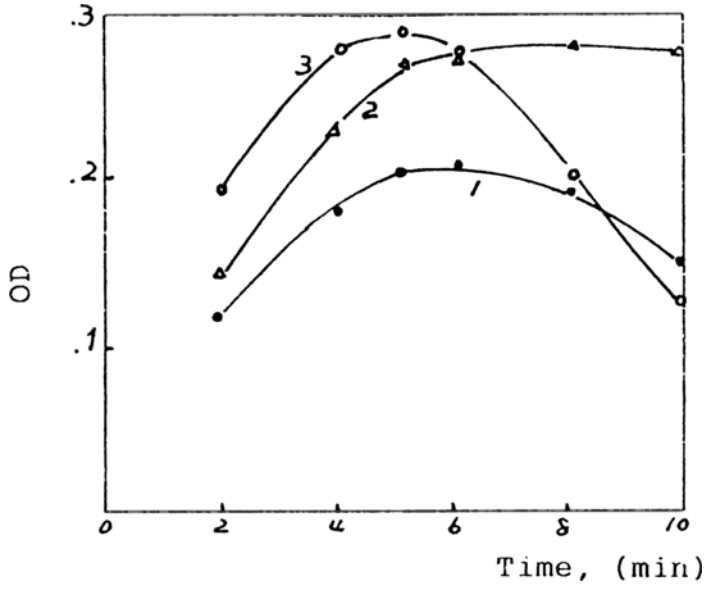


Fig. 3. Effects of FA and FM on the autooxidation of AL. 1. AL; 2. AL + FA; 3. AL + FM.

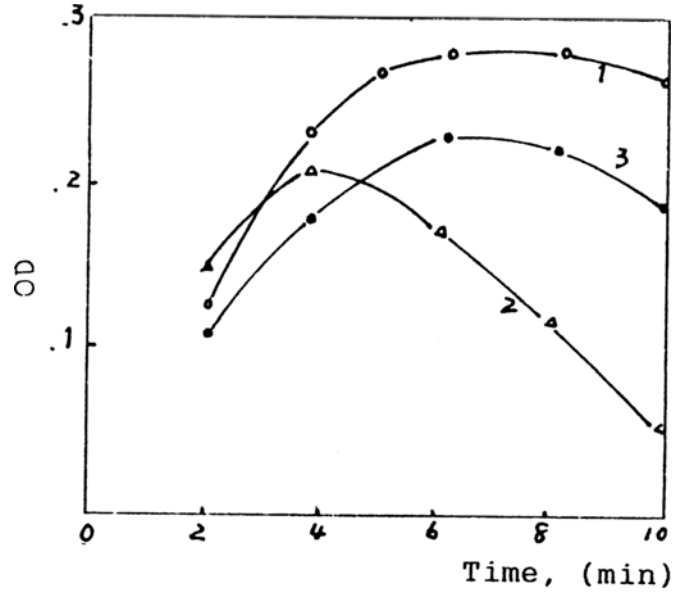


Fig. 4. Effects of Se and SOD on the autooxidation of AL in the presence of FA. 1. AL + FA; 2. AL + FA + Se; 3. AL + FA + SOD.

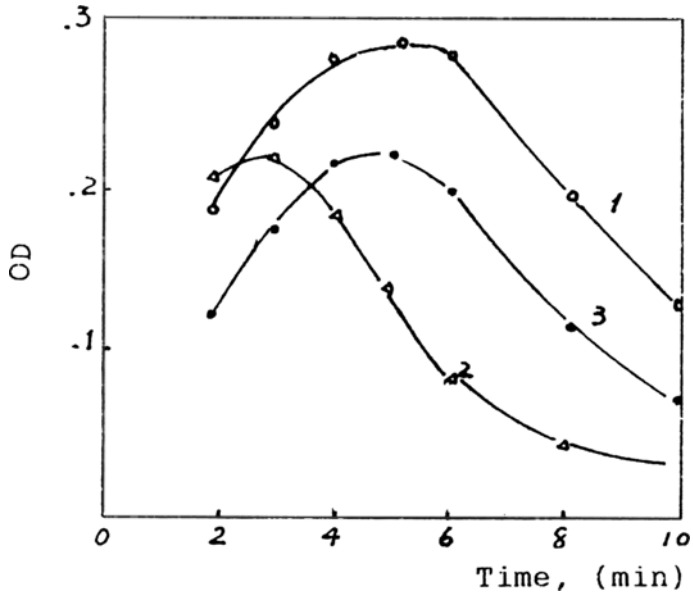


Fig. 5. Effects of Se and SOD on the autooxidation of AL in the presence of FM. 1. AL + FM; 2. AL + FM + Se; 3. AL + FM + SOD.

C, and a decrease in groups D–G, compared with group A. Obviously, FA and FM improved the ability of AL to produce $O_2^{\cdot}$. Eight minutes after the reaction had started, FM ceased its promoting effect, whereas FA was still in effect (Fig. 3). Figures 4and 5 indicate that although Se and SOD could decrease $O_2^{\cdot}$ level in FA and FM system, but a difference between Se and SOD should be observed that SOD could only remove $O_2^{\cdot}$ from the system, that corresponds with the parallel autooxidation curves of AL+FA (or FM)+SOD and AL+FA (or FM). It was also proved that selenium, as SOD, could reduce the oxyradical produced by FA and FM.

From the above experimental results, as well as previous reports, we believe that selenium is a protecting factor, as well as a free radical scavenger in KBD. The pathogenic factor could be the oxyradical generated from both FA in drinking water and mycotoxin in grain, whereas the selenium is deficient in the environment. This may explain why the supplementation of selenium, or removal of organic matters from drinking water, or changing staple food, as well as giving rich nutritional diet to children all have been found to be effective in the control of this disease. In short, the incidence of the disease decreases if the local living standard is improved, and the level of antioxidants in the body is increased.

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