Effects of Arsenic (As^{III}) on Lipid Peroxidation, Glutathione Content and Antioxidant Enzymes in Growing Pigs

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ABSTRACT : This experiment was conducted to investigate the effect of arsenic (As^{III}) on lipid peroxidation, glutathione content and antioxidant enzymes in growing pigs. Ninety-six Duroc-Landrace-Yorkshire crossbred growing pigs (48 barrows and 48 gilts, respectively) were randomly assigned to four groups and each group was randomly assigned to three pens (four barrows and four gilts). The four groups received the same corn-soybean basal diet which was supplemented with 0, 10, 20, 30 mg/kg As respectively. Arsenic was added to the diet in the form of As_2O_3. The experiment lasted for seventy-eight days after a seven-day adaptation period. Malondialdehyde (MDA) levels, glutathione (GSH) contents and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) activities were analyzed in serum, livers and kidneys of pigs. The results showed that pigs treated with 30 mg As/kg diet had a decreased average daily gain (ADG) ($p<0.05$) and an increased feed/gain ratio (F/G) ($p<0.05$) compared to the controls. The levels of MDA significantly increased ($p<0.05$), and the contents of GSH and the activities of SOD, CAT, GPx, GR and GST significantly decreased ($p<0.05$) in the pigs fed 30 mg As/kg diet. The results indicated that the mechanism of arsenic-induced oxidative stress in growing pigs involved lipid peroxidation, depletion of glutathione and decreased activities of some enzymes, such as SOD, CAT, GPx, GR and GST, which are associated with free radical metabolism. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 5 : 727-733)***

Key Words : Growing Pigs, Arsenic, Growth Performance, Lipid Peroxidation, Glutathione, Antioxidant Enzymes

INTRODUCTION

Arsenic (As) is a ubiquitous element in the environment. Weathering of rocks converts arsenic sulfides to arsenic trioxide, which enters the arsenic cycle as dust or by dissolution in rain, rivers, or groundwater (Mandal and Suzuki, 2002). It is also introduced into the environment through mine tailings, industrial wastes discharge, fertilizers, agricultural employments of pesticides, smelting of metals, and burnings of fossil fuels. Also, arsenic can enter food chain causing wide spread distribution throughout the plant and animal kingdoms.

Arsenic occurs in both organic and inorganic forms in nature but inorganic species of arsenic [As(III) and As(V)] represent a potential threat to the environment, human health, and animal health due to their carcinogenic and other effects (Singh et al., 2003). Arsenic can result in acute and chronic toxicity. The characteristics of severe acute arsenic toxicity include gastrointestinal discomfort, vomiting, diarrhea, bloody urine, anuria, shock, convulsions, coma and death (Hughes, 2002). Chronic effects are degenerative inflammatory and neoplastic changes of the skin and respiratory, haematopoietic, cardiovascular, nervous, hepatic, endocrine and renal systems (Hughes, 2002).

Arsenic is a carcinogen to both humans and animals. Inorganic arsenic is classified by the International Agency for Research on Cancer (IARC, 1980, 1987) and the US Environmental Protection Agency (EPA, 1988) as a known human carcinogen. This classification is based on epidemiological studies which show an association of exposure to arsenic and the development of cancer. The evidence for arsenic carcinogenicity in animals, however, was very limited until Ng et al. (1999, 2001) established a two year mouse model successfully to demonstrate arsenic carcinogenicity and mutagenicity.

The mechanism(s) by which arsenic induces cancer now remains poorly understood. Many different mechanisms of action have been proposed and some potential mechanisms include genotoxicity, cell proliferation, altered DNA repair and DNA methylated oxidative stress, co-carcinogenesis, and tumor promotion (Hughes, 2002). Among them, the oxidative damage is considered to play an important role in arsenic carcinogenesis. Arsenic initiates cytotoxicity by introducing oxidative damage (Lee and Ho, 1994). Oxidative stress arises when reactive oxygen species such as free radicals, lipid hydroperoxides, aldehydes, hydrogen peroxides are generated, which can react with cellular constituents such as thiols and lipids and alter the antioxidant defense systems (Keyse and Tyrell, 1989; Wang and Huang, 1994; Liu et al., 2001). In present, studies about the effects of arsenic on antioxidant defense system in pigs are few. This study was undertaken to assess the impact of the arsenic exposure on the antioxidant defense system in serum, liver and kidney of growing pigs, so as to further research the mechanism of arsenic carcinogenesis in animals and humans.

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Table 1. Ingredients of the basal diet and their nutrient levels

Item	Phases		
	30-50 kg	50-80 kg	80 kg or over
Ingredients (%)			
Corn	64.0	67.1	68.45
Soybean meal	22.0	17.0	13.0
Wheat bran	10.0	12.0	15.0
Fish meal	0.25	0.25	
Monocalcium phosphate	1.2	1.0	0.8
Limestone	1.0	1.1	1.2
Salt (NaCl)	0.35	0.35	0.35
Lysine	0.2	0.2	0.2
Mineral/vitamin premix[a]	1.0	1.0	1.0
Analyed values			
Digestible energy (Mcal/kg[b])	3.06	3.02	3.00
Crude protein (%)	17.2	15.3	14.3
Calcium (%)	0.76	0.74	0.71
Total phosphorus (%)	0.60	0.56	0.53
Total (mg/kg)	0.38	0.38	0.38

[a] Mineral/vitamin premix supplied per kilogram of complete diet: 30-50 kg: vitamin A, 4,000 IU; vitamin D_3, 500 IU; vitamin E, 40 IU; vitamin B_2, 6.2 mg; nicotinic acid, 22 mg; d-pantothenic acid, 22 mg; vitamin B_{12}, 0.02 mg; biotin, 0.15 mg; choline, 0.92 mg. Fe ($FeSO_4 \cdot H_2O$) 200 mg, Cu ($CuSO_4 \cdot 5H_2O$) 200 mg, Zn ($ZnSO_4 \cdot H_2O$) 175 mg, Mn ($MnSO_4 \cdot H_2O$) 140 mg; Se ($NaSe_2O_3$), 0.15 mg. 50-80 kg: vitamin A, 3,500 IU; vitamin D_3, 400 IU; vitamin E, 30 IU; vitamin B_2, 5.2 mg; nicotinic acid, 20 mg; d-pantothenic acid, 18 mg; vitamin B_{12}, 0.02 mg; biotin, 0.15 mg; choline, 0.77 mg. Fe ($FeSO_4 \cdot H_2O$) 175 mg, Cu ($CuSO_4 \cdot 5H_2O$) 200 mg, Zn ($ZnSO_4 \cdot H_2O$) 150 mg, Mn ($MnSO_4 \cdot H_2O$) 72 mg; Se ($NaSe_2O_3$), 0.15 mg. 80 kg or over: vitamin A, 2,500 IU; vitamin D_3, 300 IU; vitamin E, 20 IU; vitamin B_2, 4.6 mg; nicotinic acid, 20 mg; d-pantothenic acid, 15 mg; vitamin B_{12}, 0.02 mg; biotin, 0.1 mg; choline, 0.56 mg. Fe ($FeSO_4 \cdot H_2O$) 100 mg, Cu ($CuSO_4 \cdot 5H_2O$) 100 mg, Zn ($ZnSO_4 \cdot H_2O$) 120 mg, Mn ($MnSO_4 \cdot H_2O$) 60 mg; Se ($NaSe_2O_3$), 0.15 mg.

[b] Calculated analyses were based on nutrient contents of ingredients listed in NRC (1998).

MATERIALS AND METHODS

Animal treatment and sample collection

Ninety-six Duroc-Landrace-Yorkshire crossbred growing pigs (48 barrows and 48 gilts, respectively), with an average initial body weight of 30.6±1.35 kg were randomly assigned to four different treatments. Each of these groups consisted of three replications (i.e., pens) with eight pigs per replicate. The treatments received the same basal diet and supplemented with 0, 10, 20, 30 mg/kg arsenic (as As_2O_3) respectively, and three corn-soybean basal diets (Table 1) were used during experimental period (pig body weight from 30 kg to 90 kg). Pigs had access to feed and water *ad libitum*. The experiment lasted for seventy-eight days after seven days of adaptation period. The content of arsenic was 0.38 mg/kg in the basal diet. Feed intake per pen was recorded for the experimental period, and each pig was weighed at the beginning and the end of experiment to determine average daily gain (ADG), average daily feed intake (ADFI) and feed/gain ratio (F/G).

At the end of 78 days feeding trial, two pigs (one barrow and one gilt) from each pen were randomly selected to slaughter after a 24-h fast. Blood samples were centrifuged at 3,000 rpm/min for 15 min, the serum separated by blood was packed in Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -70°C until analysis. Livers and kidneys were removed and weighed from carcass within 15 min after exsanguinations. The samples of livers and kidneys were collected from the left side of these tissues, snap-frozen in liquid nitrogen, and stored at -70°C until they were analyzed.

Arsenic analyse

For blood, liver and kidney arsenic determination, wet tissue weight and volume of blood was recorded. The samples were digested with acid mixture containing nitric acid, sulfuric acid, and perchloric acid in the ratio of 6:1:1, over a regulated heater (300-500°C). The acid mixture was evaporated with occasional addition of triple distilled water and the resulting solution was employed for estimation of arsenic content. The estimation was performed by atomic absorption spectrophotometry (AAS) with hydrogen generation technique (Shimadzu AA 6501, Japan).

Biochemical analysis and enzyme assays

Liver and kidney tissues were dissected, thoroughly washed with ice-cold 0.9% NaCl, weighed, minced and homogenized (10% w/v) using 66 mmol/L chilled phosphate buffer (pH 7.0). The homogenate that was centrifuged at 1,000 g for 20 min at 4°C was used for the estimation of malondialdehyde (MDA) and reduced glutathione (GSH). The supernatant obtained was further centrifuged at 12,000 g for 20 min at 4°C to obtain the postmitochondrial supernatant, which was used for the assays of superoxide dismutase (SOD), catalase activity (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST).

The lipid peroxidation was determined by measuring the amounts of MDA via the thiobarbituric acid color reaction by the method of Wills (1966). The results were expressed as nmol MDA formed per milligram of protein (nmol/mgprot).

The GSH level was determined as described by Ellman (1959) and expressed as mg per gram of protein (mg/gprot).

The SOD activity was determined according to the method of Asada et al. (1974) by monitoring the rate of inhibition of reduction of nitroblue tetrazolium (NBT) by the enzyme. The activity of SOD was expressed as unit per milligram of protein (U/mgprot). One unit represents the amount of enzyme required to produce 50% inhibition of NBT reduction per min.

Table 2. Growth performance of pigs

Item	Supplemented arsenic (mg/kg)				SEM[1]
	0	10	20	30	
Initial wt (kg)	30.71[a]	30.51[a]	30.64[a]	30.65[a]	0.22*
Final wt (kg)	88.53[a]	87.89[a]	87.19[a]	84.70[b]	1.19*
ADG (g)	741.30[a]	735.42[a]	724.97[a]	693.02[b]	13.47*
ADFI (kg)	2.15[a]	2.10[a]	2.11[a]	2.10[a]	0.02**
F/G	2.90[a]	2.92[a]	2.91[a]	3.04[b]	0.03**

[a, b] Means within a row with different superscripts differ significantly ($p<0.05$).
[1] Standard error of mean; * n = 24 per treatment; ** n = 3 per treatment.

Table 3. Relative weight of liver and kidney of pigs (expressed as a percentage of live body weight)

Item	Supplemented arsenic (mg/kg)				SEM[1]
	0	10	20	30	
Liver (%)	1.48[a]	1.52[ab]	1.57[ab]	1.68[b]	0.06
Kidney (%)	0.33[a]	0.32[a]	0.32[a]	0.30[a]	0.01

[a, b] Means within a row with different superscripts differ significantly ($p<0.05$).
[1] Standard error of mean; n = 6 per treatment.

The CAT activity was measured spectrophotometrically by calculating the rate of degradation of H_2O_2 (Aebi, 1984) and expressed as unit per milligram of protein (U/mgprot). One unit represents 1 μmol H_2O_2 degradated per min.

The GPx activity was measured spectrophotometrically and expressed as unit per milligram of protein (U/mgprot). One unit of GPx represents 1 μmol oxidized NADPH per min (Flohe and Grunzler, 1984).

The GR activity was determined by the method of Carlberg and Mannervik (1985) and expressed as unit per gram of protein (U/gprot). One unit represents 1 μmol NADPH oxidized per min.

The GST was measured by using 1-Chloro-2-4-dinitrobenzene (CDNB) (Habig et al., 1974) and expressed as unit per milligram of protein (U/mgprot). One unit was defined as 1 μmol CDNB-GSH conjugate formed per minute.

Proteins were estimated by the method of Bradford (1976) using bovine serum albumin as the standard protein.

LPO, GSH, SOD, CAT, GPx, GR and GST activities in serum were analyzed by the above methods, the results were expressed as nmol/ml (MDA), U/L (GR), mg/ml (GSH) or units per millilitre of serum (U/ml).

Statistical analyses

The data were analyzed by comparing means according to least significant difference test using the general linear models procedure of SAS (Version 6.12). A significant level of 0.05 was used.

RESULT AND DISCUSSION

Growth performance and Relative weight of livers and kidneys

The results on changes in body weight, average daily gain (ADG), average daily feed intake (ADFI) and feed/gain ratio (F/G) of the arsenic-treated pigs are presented in Table 2. Compared to the control, ADG was decreased 6.96% ($p<0.05$) and F/G was increased 4.83% ($p<0.05$) when treated with 30 mg/kg As. However, no effect of arsenic on the ADFI of growing pigs was observed. Morrison and Chaves (1983) reported 100 mg/kg As supplementation in the weanling pigs reduced ADG and ADFI but not F/G. Male rats aged 6 wk fed diets containing 100 mg As/kg diet decreased feed and water intake and body weight gain (Yu and Beynen, 2001). Studies on broilers (Czarnecki and Baker, 1985), hens (Donoghue et al., 1994; Holcman et al., 1997, 2001) and calves (Vreman et al., 1986) also showed that excessive arsenic in diets significantly lowered animal performances.

The relative weight of livers and kidneys of the pigs are shown in Table 3. It is observed that the relative weight of liver was larger ($p<0.05$) than those of the control after arsenic exposure at concentration of 30 mg/kg and duration. However, addition of arsenic in diets had no effect on the relative weight of kidneys. Yu and Beynen (2001) reported that liver weight significantly increased but kidney weight was not affected for male rats fed 100 mg/kg arsenic diet for a period of 2 weeks. As a very active site of metabolism, the liver is the main site of arsenic intoxication, where arsenic methyltransferase enzymes mediate the methylation process with *S*-adenosylmethionine as the methyl donor and GSH as an essential co-factor. The liver can accumulate arsenic with repeated exposures. The kidney can also accumulate arsenic and is the major route of arsenic excretion. The present results showed that accumulation of excessive arsenic induced abnormalities of livers in pigs and the kidneys seemed to be less sensitive to arsenic than the livers. In humans the kidneys seem to be less sensitive to arsenic than most other organ systems (Mandal and Suzuki, 2002).

Table 4. Arsenic concentration of serum, liver and kidney of pigs

Item	Supplemented arsenic (mg/kg)				SEM[1]
	0	10	20	30	
Serum (mg/L)	0.18[a]	0.76[b]	1.25[c]	1.89[d]	0.11
Liver (mg/kg)	0.27[a]	0.59[b]	0.91[c]	1.26[d]	0.10
Kidney (mg/kg)	0.32[a]	0.73[b]	1.11[c]	1.62[d]	0.08

[a-d] Means within a row with different superscripts differ significantly ($p<0.05$).
[1] Standard error of mean; n = 6 per treatment.

Table 5. MDA, GSH and antioxidant enzymes of serum

Item[1]	Supplemented arsenic (mg/kg)				SEM[2]
	0	10	20	30	
MDA (nmol/ml)	11.28[a]	12.01[a]	12.89[ab]	13.61[b]	0.70
GSH (mg/ml)	220.85[a]	211.59[a]	200.06[ab]	190.73[b]	6.87
SOD (U/ml)	111.03[a]	105.67[ab]	96.01[bc]	87.51[c]	6.02
CAT (U/ml)	3.19[a]	3.10[a]	2.41[b]	2.32[b]	0.18
GPx (U/ml)	873.54[a]	849.34[ab]	817.47[ab]	776.21[b]	34.63
GR (U/L)	20.36[a]	19.19[a]	18.21[ab]	16.47[b]	0.92
GST (U/ml)	9.44[a]	9.01[ab]	8.36[ab]	7.97[b]	0.64

[a-c] Means within a row with different superscripts differ significantly ($p<0.05$).
[1] MDA-malondialdehyde; GSH-glutathione; SOD-superoxide dismutase; CAT-catalase activity; GPx-glutathione peroxidase; GR-glutathione reductase; GST-glutathione-S-transferase.
[2] Standard error of mean; n = 6 per treatment.

Table 6. MDA, GSH and antioxidant enzymes of livers

Item[1]	Supplemented arsenic (mg/kg)				SEM[2]
	0	10	20	30	
MDA (nmol/mgprot)	2.58[a]	2.68[a]	2.85[ab]	3.08[b]	0.13
GSH (mg/mgprot)	29.6[a]	28.25[a]	26.52[ab]	24.09[b]	1.56
SOD (U/mgprot)	72.3[a]	68.96[ab]	64.62[b]	58.49[c]	1.98
CAT (U/mgprot)	20.86[a]	18.01[ab]	17.94[b]	16.21[b]	1.34
GPx (U/mgprot)	231.60[a]	227.49[a]	213.70[a]	183.20[b]	8.28
GR (U/gprot)	1.45[a]	1.38[ab]	1.34[ab]	1.25[b]	0.08
GST (U/mgprot)	14.00[a]	13.83[a]	12.91[a]	11.09[b]	0.85

[a-c] Means within a row with different superscripts differ significantly ($p<0.05$).
[1] MDA-malondialdehyde; GSH-glutathione; SOD-superoxide dismutase; CAT-catalase activity; GPx-glutathione peroxidase; GR-glutathione reductase; GST-glutathione-S-transferase.
[2] Standard error of mean; n = 6 per treatment.

Tissue arsenic concentration

Table 4 presents arsenic concentration in blood, liver and kidney of pigs. As shown in Table 4, compared with the control, serum, liver and kidney arsenic levels of three arsenic-added groups were significantly elevated ($p<0.05$). The results demonstrated that blood and tissue concentrations of arsenic increased in response to an increase in dietary arsenic concentration.

Lipid peroxidation, glutathione and antioxidant enzymes

Table 5, 6 and 7 show the levels of Malondialdehyde (MDA), the contents of glutathione (GSH) and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) in serum, liver and kidney of pigs treated with arsenic. It reveals that arsenic treatment increased lipid peroxidation of pigs with 30 mg/kg As diets, the MDA levels in the serum, liver and kidney were elevated 20.66%,19.38% and 35%, respectively, compared to the controls ($p<0.05$). The levels of GSH content in serum, liver and kidney of pigs fed 30 mg/kg As diets decreased by 13.63%, 18.62% and 13.18% ($p<0.05$), respectively. The exposure to arsenic decreased the activities of SOD, CAT, GPx, GR and GST, especially, the concentrations of these antioxidant enzymes significantly lower when added 30 mg/kg As in diet than those of the controls ($p<0.05$).

Studies by Ramos et al. (1995) demonstrated a tendency for a positive correlation between arsenic concentration and lipid peroxidation level in liver, kidney and heart of rats following acute exposure to arsenic. Flora et al. (2002) reported that GaAs induced lipid peroxidation in blood, liver and kidney of rats. This study indicated that the action of lipid peroxidation was increased by high arsenic level and duration. Arsenic induced MDA production could be due to the impairment of cells' natural protective system and could be directly related to the GSH depletion.

Table 7. MDA, GSH and antioxidant enzymes of kidney

Item[1]	Supplemented arsenic (mg/kg)				SEM[2]
	0	10	20	30	
MDA (nmol/mgprot)	0.80[a]	0.88[a]	0.93[ab]	1.08[b]	0.07
GSH (mg/mgprot)	25.57[a]	24.91[ab]	24.28[ab]	22.20[b]	1.45
SOD (U/mgprot)	71.20[a]	69.90[a]	54.27[b]	52.68[b]	3.14
CAT (U/mgprot)	24.59[a]	22.01[ab]	19.03[bc]	15.80[c]	1.63
GPx (U/mgprot)	207.20[a]	180.42[b]	175.08[bc]	159.00[c]	7.2
GR (U/gprot)	2.57[a]	2.46[a]	2.28[ab]	1.98[b]	0.14
GST (U/mgprot)	9.03[a]	8.26[ab]	7.70[b]	7.10[b]	0.52

[a-c] Means within a row with different superscripts differ significantly ($p<0.05$).

[1] MDA-malondialdehyde; GSH-glutathione; SOD-superoxide dismutase; CAT-catalase activity; GPx-glutathione peroxidase; GR-glutathione reductase; GST- glutathione-S-transferase.

[2] Standard error of mean; n = 6 per treatment.

Glutathione is well known for its pivotal role on the intrinsic antioxidant system of mammalian cells. The present study showed that relative high arsenic level and duration depleted significantly GSH content in serum, liver and kidney of growing pigs. Maiti and Chatterjee (2001) reported that after an acute arsenic exposure GHS concentration was significantly depleted. The effect of GSH depletion on the mutagenicity of arsenic has also been examined by many scholars (Hei et al., 1984; Xu et al., 1999; Liu et al., 2003).

A decrease in the activity of SOD can be owed to an enhanced superoxide production during arsenic metabolism (Searle and Wilson, 1980). SOD catalyzes the dismutation of superoxide anions and prevents the subsequent formation of hydroxyl radicals (Imlay et al., 1988). In the present study, the decreased SOD activity in serum, liver and kidney of pigs suggested that the accumulation of superoxide anion radical might be responsible for increased lipid peroxidation following arsenic treatment (Maiti and Chatterjee, 2000). That arsenic can suppress SOD activity was also observed in larvae of an insect (Zaman et al., 1995).

The superoxide radical also inhibited the activity of catalase (Kono and Fridovich, 1982). Exposure to arsenic decreased the CAT activity (Kirkman and Gaetani, 1984). Arsenic inhibited the catalase activity on human fibroblast cells (Lee and Ho, 1995). Wang and Huang (1994) showed that arsenic induced increase of micronuclei by overproduction of H_2O_2. CAT catalyzes the removal of H_2O_2 formed during the reaction catalyzed by SOD. In the present study, the decreased CAT activity indicated that exposure to arsenic result in impaired ability to detoxify H_2O_2 via catalase and accumulation of H_2O_2 in serum, liver and kidney of pigs.

The depletion of GSH level in serum, liver and kidney of pigs after exposure to arsenic may be accounted by the decrease in GR activity. Arsenic can inhibit the activity of GR (Cunningham et al., 1994; Styblo et al., 1997). The inhibition may be due to the interaction of trivalent arsenic with critical thiol groups in GR molecules.

GPx reduces lipid hydroperoxides into lipid alcohols, this enzyme is coupled with GR. GSH serves as a substrate for GPx. In this study, the decreased GPx activities in serum, liver and kidney of pigs were due to the decrease in the levels of GSH and increase in the levels of LPO during arsenic exposure (Wang et al., 1997).

GST can remove free radicals and its levels can reflect the antioxidant capacity in the body. The present studies showed that exposure to arsenic decreased GST activities in serum, liver and kidney of pigs. Falkner et al. (1993), however, reported that acute arsenic treatment caused an increase in GST activity of kidney but not of liver and lung in guinea pig. Maiti and Chatterjee (2001) also reported that after an acute arsenic exposure GST activity significantly decreased in liver while GST increased in kidney in rats. This indicates that some of the responses of cellular protective mechanisms of tissue against arsenic insult may also be different depending on nature, the dose, duration and route of arsenic exposure and the stage of life at which arsenic were administered.

IMPLICATION

The present study shows that high arsenic can markedly alter MDA, GHS content and some enzymes activities associated with free radical metabolism in serum, livers and kidneys of growing pigs. The findings indicate that high-arsenic and duration can induce oxidant stress in growing pigs. The mechanism of arsenic on antioxidant defense system in the body seem to involve in lipid peroxidation, depletion of glutathione and decreased activities of some enzymes, such as SOD, CAT, GPx, GR and GST, which associated with free radical metabolism.

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