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Influence of combined antioxidants against cadmium induced testicular damage

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Abstract

Acute effects of cadmium (Cd) and combined antioxidants were evaluated in Sprague–Dawley rat testes. The rats were subdivided into four groups. Cadmium chloride (2 mg/kg day) injected intraperitoneally during 8 days. Vitamin C (250 mg/kg day), vitamin E (250 mg/kg day) and sodium selenate (0.25 mg/kg day) were pretreated by gavage in both of control and cadmium injected rats. Testis lipid peroxidation and glutathione levels were determined by spectrophotometrically. In Cd treated rats, lipid peroxidation levels were increased and glutathione levels were decreased and combined antioxidants treatment was effective in preventing of lipid peroxidation and normalizing glutathione. In Cd treated animals, the degenerative changes were observed, but not observed in the administrated rats with Cd and antioxidants under the light microscope. Proliferating cell nuclear antigen, metallothionein and caspase-3 activities were evaluated by immunohistochemically. Proliferation activity was not seen in the spermatogonial cells of cadmium treated testis. Treatment with antioxidants in cadmium administrated testis leads to pronounced increase in proliferation activity. Cytoplasmic caspase-3 activity was determined in the spermatogenic cells but not spermatogonia in treatment of antioxidants with Cd. In control and treated with antioxidants animals, metallothionein expressions were localized in the cells of seminiferous tubules, although the expression only was observed in the interstitial cells of cadmium treated rats. Results demonstrated beneficial effects of combined vitamin C, vitamin E and selenium treatment in Cd toxicity.

Keywords: Antioxidants; Cadmium; Rat; Testis

1. Introduction

Cadmium (Cd) is one of the most toxic industrial and environmental heavy metal that has been known to damage renal, hepatic, respiratory and reproductive system (WHO, 1992). The prominent toxic effects of Cd were recognized very well in testes (Nolan and Shaikh, 1986). Various mechanisms have been suggested to explain Cd induced cellular toxicity. Reactive oxygen species enhance lipid peroxidation, altered antioxidant system, DNA damage, altered gene expression and apoptosis (Stohs et al., 2001). In agreement with these suggestions, the increase of lipid peroxidation and especially depletion of glutathione were reported in cadmium toxicity of testis (Yiin et al., 1999). Caspase-3 is an effector and critically caspase for detection of cells to apoptosis (Kamada et al., 2005). Caspase-3 activity was measured in Cd induced cells after activation of initiator caspases (Kondoh et al., 2002). Cell death occurs with necrosis or apoptosis with dose dependent in Cd induced testis (Lopez et al., 2003; Gupta Sen et al., 2004a). Spermatogonia are located in the basal compartment of seminiferous tubules and are functionally characterized by repeated mitotic divisions in spermatogenesis. Many defence mechanisms are implicated with Cd induced oxidative damage. Among of these mechanisms, the antioxidants such as ascorbic acid, α-tocopherol and selenium play a role as free radical scavenger (Yoshiro et al., 2003; Fang et al., 2002). It was reported that testis could be protected from toxic effects of Cd remarkably by mainly antioxidants treatment (Yiin et al., 1999; Gupta Sen et al., 2004a,b). Also, metallothionein (MT) is a metal binding antioxidant protein, which suggested participating to

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the protection mechanism in cadmium toxicity (Park et al., 2001).

In the light of information above, we undertook the present study to investigate whether treatment with vitamin C, vitamin E and selenium protects rat testis by reducing oxidative stress in response to daily repeated toxic Cd dose for acute term. Our results indicate Cd caused an increase lipid peroxidation and a decrease in glutathione levels in testis. Cd treatment also resulted with necrotic cell death and change of MT localization. Antioxidants treatment decreased lipid peroxidation and increased glutathione. In addition, proliferation activity and MT localization was detected similar with controls. Cytoplasmic caspase-3 activity was determined in spermatogenic cells, except spermatogonia. These observations suggest that combination of vitamin C, vitamin E and selenium may have significance in the prevention of acute Cd toxicity in rat testis.

2. Materials and methods

2.1. Animals and experimental design

Fifty-nine adult male Sprague–Dawley, 6.5–7 months old rats from DETAM (Istanbul University Centre for Experimental Medical Research and Application) were used in this study. The experiments were reviewed and approved by Local Institute's Animal Care and Use Committees. The animals were randomly divided into four groups: Group I: ($n = 16$) were intact control animals. Group II ($n = 16$) was control rats given vitamin C (250 mg/kg day) + vitamin E (250 mg/kg day) + sodium selenate (0.25 mg/kg day). Group III ($n = 17$) was the animals given only cadmium chloride (2 mg/kg day $CdCl_2$, intraperitoneal) for 8 days and group IV ($n = 10$) was rats given vitamin C + vitamin E + selenium + Cd in same dose and time. The animals were treated by antioxidants 1 h prior to treatment with cadmium every day. Vitamin E was dissolved in sunflower oil. Selenium, vitamin C and Cd were dissolved in distilled water. The antioxidants were given to rats by gavage and Cd was given intraperitoneally. Effective antioxidant doses, treated time and route were determined with a pilot study according to our previous results (Koyuturk et al., 2004; Ozdil et al., 2004). Cd administration dose was used according to reports (WHO, 1992; Gupta Sen et al., 2004a). On the 9th day of experiment, all of the animals were fasted overnight and then sacrificed under ether anesthesia.

2.2. Biochemical study

In this study, biochemical investigation was made in testicular tissues. For biochemical analyses, tissue samples of testes were washed with physiological saline and kept frozen until the day of the experiment. Tissues were homogenized in cold 0.9% NaCl in a glass homogenizer to make up 10% homogenate (w/v). Homogenates were centrifuged and the clear supernatants were used for protein, lipid peroxidation (LPO) and glutathione (GSH) analyses.

LPO levels in testicular homogenates were estimated by the method of Ledwozwy et al. (1986). In brief, the adducts formed following boiling tissue homogenate with thiobarbutiric acid is extracted with *n*-butanol. The difference in optical density at 532 nm is measured the testicular malondialdehyde (MDA) content as a measure of TBARS, which is undertaken as an index of lipid peroxidation. Results were expressed as nmol MDA/mg protein.

Reduced glutathione (GSH) levels were determined according to the method by Beutler using Ellman's reagent (Beutler, 1975). The procedure is based on the reduction of Ellman's reagent by SH groups to inform 5,5′-dithiobis(2-nitrobenzoic acid) which has an intense yellow color that is measured spectrophotometrically at 412 nm using Shimadzu spectrophotometer. Results were expressed as nmol GSH/mg protein.

The protein content in the supernatants was assayed by the method of Lowry et al. (1951) using bovine serum albumin as a standard (Lowry et al., 1951).

2.3. Histological study

On the 9th day of the experiment, the animals sacrificed under ether anesthesia, 1 day after last treatment with cadmium. The pieces of testes tissues were fixed in Bouin's fixative and passed from increasing alcohol and embedded in paraffin. Sections of 5 μm thickness were stained by hematoxylin–eosin and examined under Olympus CX41 light microscope.

2.4. Immunohistochemical study

Slides were deparaffinized in toluene and hydrated in ethanol series. For antigen retrieval, the slides were pressure-cooked in 0.01 M citrate buffer (pH 6). Then Histostatin Plus (Zymed Laboratories, USA) broad spectrum kit of the streptavidin–biotin system was applied. Proliferation activity was assessed with proliferating cell nuclear antigen (PCNA) antibody at room temperature at 1:50 dilution (Lab Vision, UK). Caspase-3 activity was assessed with monoclonal antibody (Lab Vision, UK) at also room temperature at 1:60 dilutions. Metallothionein expression was evaluated by using antibody against metallothionein protein (Zymed Laboratories, USA) for overnight at 4 °C at 1:60 dilutions. Sections were incubated with biotinylated secondary antibody then incubated with the streptavidin–peroxidase conjugate. The enzyme activity was developed using aminethylcarbazole. Negative control sections were prepared by substituting the primary antibodies with phosphate-buffer saline.

2.5. Statistical analysis

The results were evaluated using an unpaired *t*-test and analysis of variance (ANOVA) using the NCSS statistical computer package (Hintze, 1986).

3. Results

3.1. Biochemical results

The mean testes values LPO and GSH levels of four experimental groups are presented in Table 1. The LPO levels were significantly increased in cadmium groups as compared to the other groups ($P_{\text{ANOVA}} = 0.0001$). Testes LPO levels in cadmium group were significantly increased as compared to the control group ($^{a}P_{t\text{-test}} = 0.0001$). Administration of vitamin C, vitamin E and selenium caused a significant decrease in the LPO levels in the cadmium administrated rats ($P_{t\text{-test}} = 0.0001$), but caused a significant increase in LPO levels in the control rats ($P_{t\text{-test}} = 0.007$).

A significant difference in the testes GSH levels of four groups was observed ($P_{\text{ANOVA}} = 0.0001$). Testes GSH levels in cadmium group were significantly decreased as compared to the control groups ($^{a}P_{t\text{-test}} = 0.0001$). Administration of vitamin C, vitamin E and selenium caused a significant increase in GSH levels in cadmium treated rats ($P_{t\text{-test}} = 0.0001$).

3.2. Histological results

Histopathologic evaluations of testicular tissue were examined in the sections dyed hematoxylin–eosin. In Cd administrated animals, the seriously damage was detected in the integrity of spermatogenic cells of seminiferous tubules and also examined an increase of interstitial tissue. In addition, necrotic cells and debris were examined in seminiferous tubules. Degenerative changes were remarkably ameliorated and necrotic cell death prevented with antioxidants treatment in Cd induced testicular injury.

Table 1
Effects of cadmium and vitamin C + vitamin E + selenium on the levels of LPO and GSH in the testes

Groups	n	LPO* (nmol MDA/mg protein)	$P_{t\text{-test}}$	GSH* (nmol GSH/mg protein)	$P_{t\text{-test}}$
Control	16	1.01 ± 0.50	0.007	43.44 ± 5.42	0.024
Control + vitamin C + vitamin E + Se	16	1.42 ± 0.30		47.97 ± 5.33	
Cadmium	17	4.70 ± 0.80[a]	0.0001	20.26 ± 4.78[a]	0.0001
Cadmium + vitamin C + vitamin E + Se	10	2.37 ± 0.73		41.91 ± 3.65	
P_{ANOVA}		0.0001		0.0001	

n = number of animal.
[a] $P_{t\text{-test}} = 0.0001$ vs. control group.
* Mean ± S.D.

3.3. Immunohistochemical results

3.3.1. Proliferating cell nuclear antigen (PCNA) expression

Proliferation activities were observed in spermatogonial series of seminiferous tubules with PCNA. Proliferation activities were shown in spermatogonia of control rats, which were intact, and treated antioxidants (Fig. 1A). In the rats given cadmium, proliferation activity was detected, neither in the seminiferous tubules nor in interstitial tissues of testes (Fig. 1B). However, in the rats treated with both of Cd and antioxidants, proliferation activities were observed in seminiferous tubules, which were similar with controls (Fig. 1C).

3.3.2. Caspase-3 activity

Caspase-3 activity was assessed with monoclonal antibody in testicular tissues. Expression was not observed in intact and antioxidants treated control groups (Fig. 2A). Also, caspase-3 expression was not examined in testes of cadmium treated rats (Fig. 2B). Cytoplasmic caspase-3 activity was detected in all spermatogenic cells of seminiferous tubules but not in spermatogonia of the rats treated with Cd and antioxidants (Fig. 2C).

3.3.3. Metallothionein expression

MT expressions were observed in cells of seminiferous tubules which including Sertoli cells and spermatogenic cell lines. In testicular tissue of control rats with treated antioxidants, MT expression was similar with intact control (Fig. 3A). MT expression was detected only in interstitial cells in the testes of animals given cadmium (Fig. 3B). However, MT expression was detected in seminiferous tubules of the rats treated with cadmium and antioxidants which has similar appearance with both of controls (Fig. 3C).

4. Discussion

Cadmium is a toxic metal, which promotes an oxidative stress and contributes to the development of serious degenerative

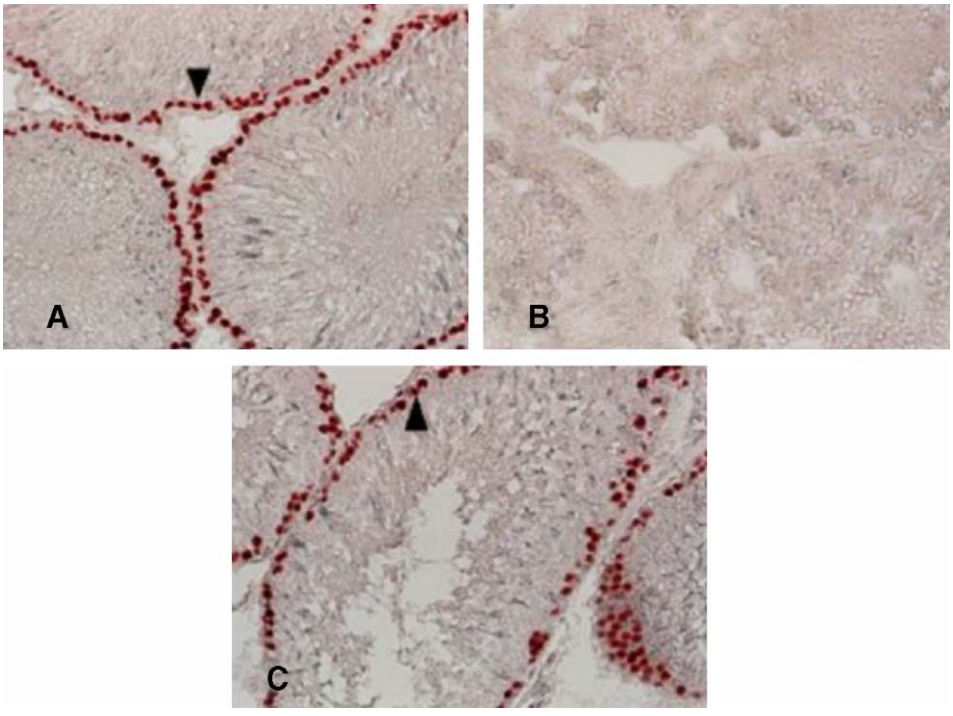

Fig. 1. Proliferation activity in testes as shown by PCNA staining in nuclei. Proliferation was seen in spermatogonial series of controls (▼) (A) but proliferation activity was not observed in cadmium induced testes (B). Proliferation activity was similar with controls in the group given cadmium and antioxidants (▲) (C) ×540.

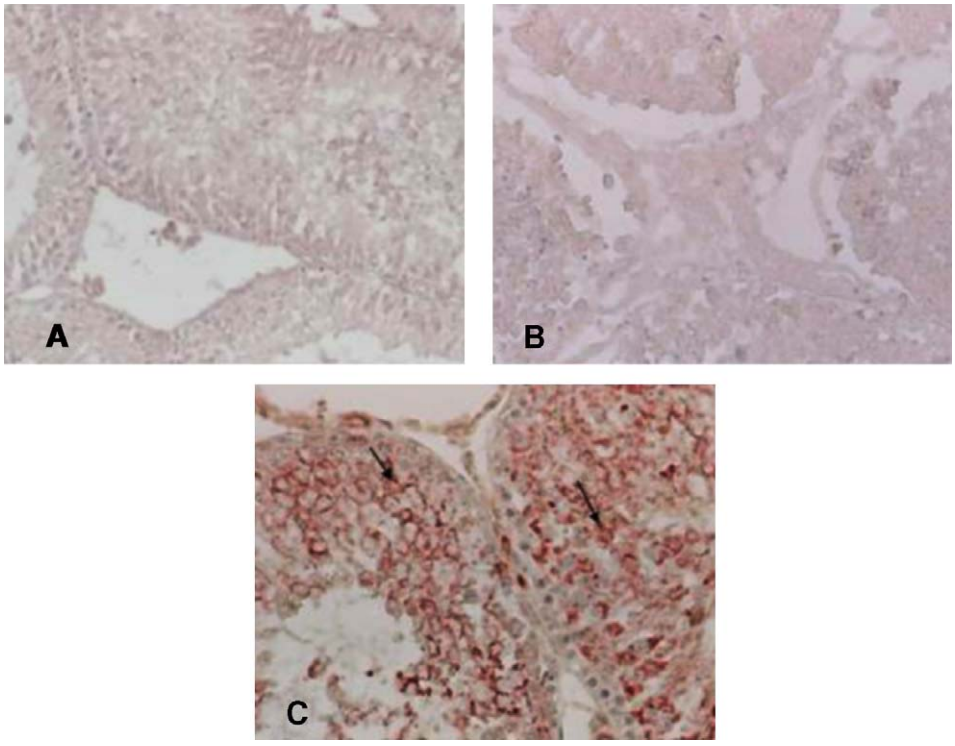

Fig. 2. Caspase-3 activity was not observed in controls (A) and cadmium induced testes (B). Cytoplasmic caspase-3 activity was seen in testes of given cadmium and antioxidants (→) (C) ×540.

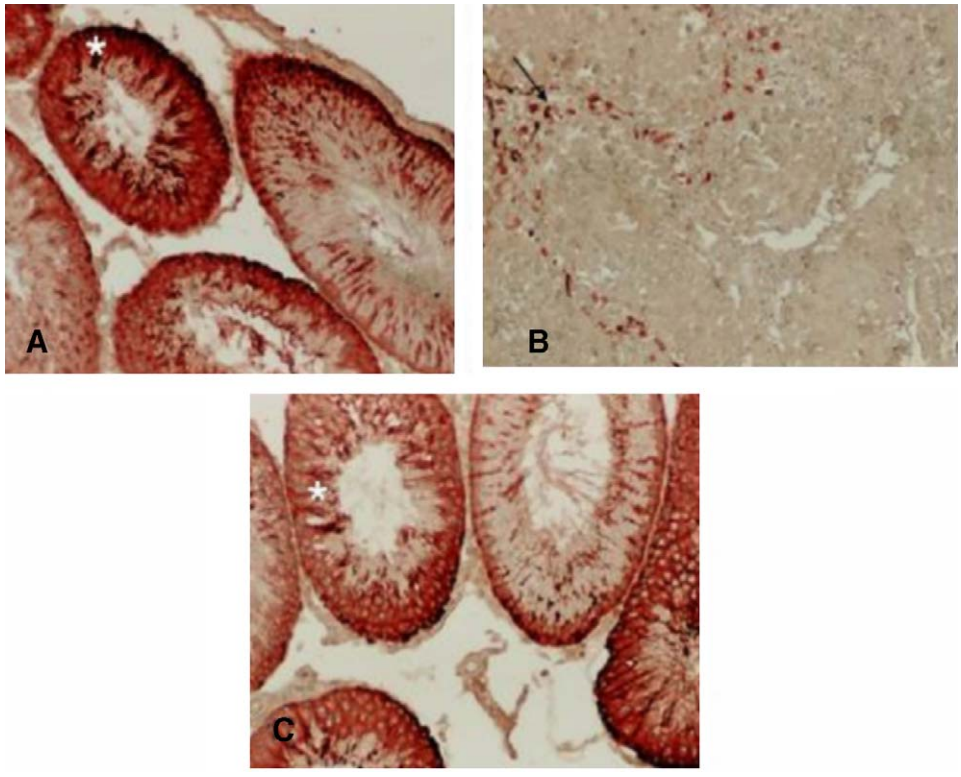

Fig. 3. Metallothionein expressions were localized in seminiferous tubules of controls (*) (A) and given cadmium and antioxidants (*) (C). In the testes of cadmium induced animals, metallothionein expression was localized in interstitial tissue (→) (B) ×270.

changing in several tissues. It is well known that testis is very sensitive to acute Cd toxicity. In the present study, Cd induced testicular tissues showed a significant increase in LPO levels. This result is agreement with various reports demonstrating that Cd induces oxidative stress by increasing LPO level and by altering antioxidative status (El-Demerdash et al., 2004; Stohs et al., 2001; Yiin et al., 1999). GSH is most abundant cellular thiol, which serves to protect against various forms of metal toxicity as well as Cd (Dalton et al., 2004). GSH is also known as free radical scavenger and potent inhibitor of LPO (Arthur, 2000). It was reported that Cd is caused GSH depletion with stimulation free radical production (Bagchi et al., 1996). It was also demonstrated that GSH levels might be direct or indirect targets of Cd exposure in testicular tissue. The current results were in conjunction with previous studies (Sugawara and Sugawara, 1984). Selenium is well known antioxidant, which has a protective effect in Cd metabolism and complex responses of glutathione dependent enzymes (Rana and Verma, 1996). Yiin et al. (1999) showed that selenium treatment reduced LPO levels in Cd induced testis. Also, ascorbic acid is an antioxidant, which decreases endogenous lipid peroxidation, oxidative protein damage and in regenerated reduced form of α-tocopherol (Gupta Sen et al., 2004a). In addition, synergistic action of combined vitamin E and selenium or ascorbic acid and vitamin E was reported (Burk, 1983; Weiss, 1986). Reduced lipid peroxidation levels reported with supplementation of ascorbic acid in Cd induced testis (Gupta Sen et al., 2004a). Rana and Verma (1996) were reported antioxidative effect of α-tocopherol in preventing oxidative stress against cadmium induced injury. Our results indicate that administration of combined form of vitamin E, vitamin C, and selenium was effective reduced LPO level and increased GSH level.

The current study obviously shows that histopathologic changes in testis are associated with the increasing of LPO and reducing GSH in conjunction by previous reports (Nolan and Shaikh, 1986; Liu et al., 2001). Current microscopic examination indicates Cd induced degenerative changes are prevented with combined antioxidants treatment. PCNA is elevated in the nucleus during late G_1 and S phases and play a fundamental role in the initiation of cell proliferation (Maga and Hübscher, 2003). In Cd induced animals, we have not detected proliferation in spermatogonial cells of seminiferous tubules with PCNA. However, proliferation activities of spermatogonia were preserved in the rats treated with antioxidants. Spermatogonia, which are primitive germ cells, are transformed into sperms after mitotic divisions (Olive and Cuzin, 2005). Therefore, continuance of proliferation in spermatogonial cells is important to support protective effects of antioxidants against Cd induced testicular injury. Reactive oxygen species have been highlighted, as a mediator factor of apoptotic cell death in Cd induced oxidative stress (Stohs et al., 2001). It was suggested that activation of caspase-8 and -9 are initiator and finally signal converges to caspase-3 in Cd induced apoptosis (De Faverney Risso et al., 2004). Apoptosis is a multi-stage type of cell death and caspase-3 activation plays a key role during this process. Subcellular localization of caspase-3 defined in apoptotic pathway. It was suggested that cytoplasmic caspase-3 translocated into nucleus after induction of apoptosis (Kamada et al., 2005). When caspase-3 has been activated, apoptotic process is irreversible and cause to nuclear morphological changes. Apoptotic and/or necrotic cell deaths were reported in spermatogenic epithelium with dose dependent in Cd toxicity (Gupta Sen et al., 2004a). Our light microscopic results support necrotic cell death in cadmium induced rats. Interestingly, caspase-3 activity was demonstrated in the cytoplasm of spermatogenic cells, except spermatogonial series of the testes in the rats given Cd and antioxidants. Administration of antioxidants preserved spermatogonia as reserve cells and caused cytoplasmic caspase-3 expression in other spermatogenic series of Cd induced testes. This process may be result with induction of apoptotic cell death then the translocation of cytoplasmic caspase-3 into nucleus but not affect the reproductive capacity throughout the life. Although, fertilising potential of sperms that originate from affected spermatogonial cells need to investigate with further studies. Our result indicated antioxidants treatment reduces oxidative stress derived from administration of toxic dose Cd. This may suggest that antioxidants treatment may be trigger a signalling pathway in affecting spermatogenic cells by Cd. Therefore, apoptotic pathway may start via caspases. There has been no reports indicating an action between antioxidants and caspase-3 or other caspases in Cd induced cell death.

Metallothionein (MT) is important antioxidant protein in the cellular defence against Cd toxicity. MT expression was reported in testis at higher levels than in other target organs such as liver (Suzuki et al., 1998; Cyr et al., 2001). A number of studies suggested that MT expression is mainly in Sertoli cells and Leydig cells but not in spermatogenic cells (Danielson et al., 1982; Nolan and Shaikh, 1986; Ren et al., 2003). Another studies reported that MT expression was localized in spermatogenic cell, spermatozoa and Sertoli cells under physiological conditions, but not in interstitial cells (Tohyama et al., 1994; Nishimura et al., 1990). The result of present study supports the localization of MT expression in seminiferous tubule but not in interstitial cells under physiological conditions and administration with combined antioxidants. In Cd induced testes, MT expression was observed only in interstitial cells. MT expression of interstitial cell in Cd induced testis might be related with accumulation side of this metal. Concentration of Cd had been shown in the interstitial tissue by autoradiography (Berlin and Ullberg, 1963). It was also suggested that interstitial cells might serve to sequestration and detoxification of Cd (Danielson et al., 1982).

Taken together, our findings indicate treatment of combined vitamin C, vitamin E and selenium inhibit remarkably testicular damage in cadmium induced rats. Combination form of antioxidants might be very useful in protection of testis against cadmium toxicity.

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