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Effect of the Vitamin E on Expression of Apoptosis-Related Proteins in Immobilized Rat Testes

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Abstract: The aim of this study was to investigate effect of the vitamin E on changes in Bax and Bcl-2 expression in the rat testes during immobilisation stress. The animals were divided into three groups. The rats in group I were immobilised for 6 h on 5 consecutive days. The rats in group II were daily injected vitamin E (100 mg kg^{-1}) for 5 days during immobilisation. Control rats were allowed to move freely. The percentage of Bax immunpositive spermatogonia in stress group was significantly higher than those in control groups ($p<0.05$) but Bcl-2 was not significantly ($p>0.05$). The percentages of Bax and Bcl-2 immunpositive cells in the vitamin E treated group was decreased than the immobilization stress group. It was observed that Bax and Bcl-2 expression were peak level (+) in the spermatogonia of the control group. In this group, Bax and Bcl-2 expression were not determined mostly in the seminifer tubules. Neither Sertoli cells nor spermatids showed immunoreaction. Bax immunoreactivity was observed at strong level (+++) in both spermatogonia and intersititial cells of immobilised rats. In this group, Bcl-2 protein was expressed at peak level (+) in the spermatogonia. Bax expression was decreased in vitamin E treated group and at peak level (+) immunreaction was showed in the spermatogonia and intersitisyal cells. Findings of this study show that the immobilisation stress increases apoptosis and vitamin E decreases Bax and Bcl-2 expression that are apoptosis-releated proteins in the immobilised rat testes.

Key words: Bax, Bcl-2, immobilisation, testes, vitamin E, spermatogonia

INTRODUCTION

Apoptotic germ cell death is an important mechanism in testicular development (Ketola *et al*., 2003) and elimination of germ cells under normal physiological and pathological conditions (Sinha and Swerdloff, 1999; Richburg, 2000).

Apoptosis is regulated by different molecular mechanism in the testes (Koji, 2001). Bcl-2 family of proteins is widely recognised as group of apoptotic regulators (Lin *et al*., 1999; Oldereid *et al*., 2001). Bax and Bcl-w deficient male mice are infertile due to disrupted spermatogenesis (Richburg, 2000).

It has been show that testicular germ cell apoptosis increases in experimental cryptorchidism (Yin *et al*., 1997), local heat stress (Yamamato *et al*., 2000), immobilisation stress (Akinbami *et al*., 1999; Almeida *et al*., 2000; Demura *et al*., 1989; Pellegrini *et al*., 1998; Saurez *et al*., 1996; Yazawa *et al*., 1999), vasectomy (Shiraishi *et al*., 2001), ischemia/reperfusion (Lysiak *et al*., 2000), chronic cigarette smoking (Rajpurkar *et al*., 2002) and x-ray (Beumer *et al*., 2000), Ethane Dimethanesulphonate (EDS) (Taylor *et al*., 1998, 1999; Woolveridge *et al*., 2001), 2-bromopropane (Yu *et al*., 2001), cisplatin, chronic alcohol (Zhu *et al*., 2000) treatment.

Immobilisation stress decreases the activities of catalase, glutathione peroxidase, glutathione transferase and glutathione reductase in the interstitium of testes. Stress-induced stimulation of the testicular Nitric Oxide (NO) signalling pathway leads to inhibition of both steroidogenic and antioxidant enzymes (Kostic *et al*., 2000).

It is well known that vitamin E had antioxidant activity in the different stress conditions. However to the knowledge, there is no study dealing with the relationship between immobilisation stress-induced apoptosis and vitamin E.

In this study, we aimed to investigate the effect of vitamin E on expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins in the rat testes during immobilisation stress by immunohistochemical methods.

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MATERIALS AND METHODS

Adult male-Wistar rats weighing about 250-300 g each (n = 18) were used in this study. The animals were maintained at constant temperature (21±2°C) and humidity (50±5%) on a 12 h light/12 h dark cycle (light on from 07.00-19.00 h). They were housed in plastic cages (six rats per cage) and fed with standard pellet food and tap water *ad-libitum*. The animals were divided into three groups. The rats in group I were allowed to move freely and designated as control. The rats in group II were immobilised for 6 h (from 09.00-15.00 h) on 5 consecutive days, by securing them on their backs to a board with adhesive tapes. The rats in group III were daily injected vitamin E (DL-α-tocopherol succinate, 100 mg kg^{-1}, Sigma, St. Louis, MO) during immobilisation for 6 h on 5 consecutive days. At the end of study period, the rats were weighed and killed by decapitation. The testes were removed, dissected free from adjacent connective tissue and weighed. Organ weights were expressed as g/100 g body weight. The testicular tissue was fixed in the Bouin's solution, dehydrated in alcohol, embedded in paraffin.

Immunohistochemistry: Immunohistochemical staining was performed using the streptavidin-biotin complex method. The 5 μm thick sections were cut onto positively charged slides. These were deparafinized in xylene and rehydrated in graded ethanol. After performing proteolysis with 0.1% trypsin (type II, crude from porcine pancreas and Ca Cl_2, Sigma) at 37°C for 30 min slides were pre-treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Then slides were rinsed with deionized water and placed in Phosphate-Buffered Saline (PBS) for at least 5 min. specimens were incubated for 30 min in normal goat serum (Dako, diluted 1:30). Slides were then incubated with primary mouse antibody (Bax and Bcl-2, Dako, USA) at 1:100 dilution for 30 min at room temperature.

After washing with two changes of PBS for 5 min each, slides were incubated for 30 min with biotin-conjugated goat anti-rat Ig G seconder antibody. After washing with PBS, slides were incubated with streptavidin-biotin-peroxidase complex for 30 min. Staining was completed with 3-Amino 9-Ethyl Carbazole (AEC) for 5 min and slides were counterstained with Mayer's Hematoxylin, cover-slipped permount. Bax and Bcl-2 immunohistochemical reaction was determined as (0) negative, (+) peak level, (++) mild level, (+++) strong level under the light microscopy.

Statistical analysis: The body and testes weights were expressed as the median±Standard Deviation (SD). All data were analysed by the non-parametric Kruskal-Wallis test and subsequent individual comparisons by the Mann-Whitney U-test. A $p<0.005$ were considered to be statistically significance.

RESULTS

In the present study, we observed that body weights of rats in immobilized and vitamin E treated groups were decreased significantly compared with the control groups ($p<0.05$). While the decrease was 26% in immobilized group, it was much less in vitamin E treated group (17%). However, there was no a significant difference between testes weights of all the groups ($p>0.05$, Table 1).

The Bax and Bcl-2 postive stainings were mostly observed in the spermatogonia of control and experimental groups. The percentage of Bax immunpositive spermatogonia in the immobilization stress group was significantly higher than those in control groups ($p<0.05$), but Bcl-2 was not significantly ($p>0.05$).

The percentage of Bcl-2 immunpositive cells in the immobilization stress group was similar to the control group. The percentages of Bax and Bcl-2 immunpositive cells in vitamin E treated group were decreased than the immobilization stress group. It was observed that decreased in Bax percentage was significant ($p<0.05$) but Bcl-2 was not significantly (Table 2).

Degeneration was not determined in the seminiferous epithelium of all the groups. It was observed that Bax and Bcl-2 expression were peak level (+) in the spermatogonia of the control group. Neither Sertoli cells nor spermatids shoved immunreaction (Fig. 1). Bax immunostaining was observed at strong level (+++) in both spermatogonia in the seminiferous tubules (Fig. 2) and intersitisyal cells

Table 1: Body and testes weights in control and experimental groups (values are mean±SD)

Groups	n	Body weight (g)	Testicular weight (g)
Control	6	287.5±35.4	0.454±0.05
Immobilised group	6	210.0±16.2*	0.455±0.05
Vitamin E treated group	6	237.5±23.6*	0.50±0.002

Table 2: Ouantities of Bax and Bcl-2 immunopositive cells in control and experimental groups (values are mean±SD)

Groups	n	Bax	Bcl-2
Control	6	1.00±0.26	2.17±0.31
Immobilised group	6	3.17±0.48*	2.13±0.28
Vitamin E treated group	6	1.80±0.25	1.83±0.40

$p<0.05$ compared to the other groups

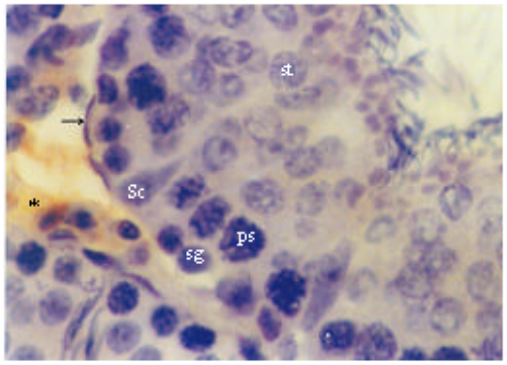

Fig. 1: Bax immunoreactivity seen at negative level (0) in the myoid cell (→), spermatogonia (sg), primary spermatocytes (ps), spermatid (st) and Sertoli cell (Sc) in the seminiferous tubules and at mild level (++) in the interstitium (*) of control rat testes. Magnification, x40

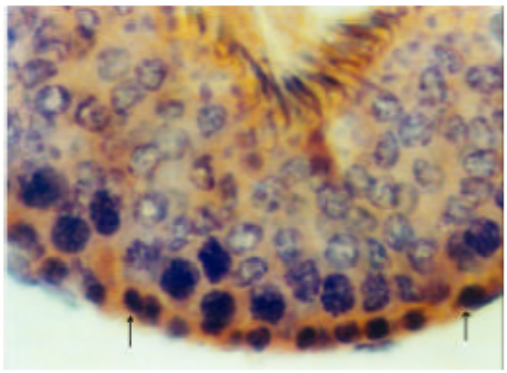

Fig. 2: Immunohistochemical staining of Bax observed at strong level (+++) in the spermatogonia (→) of immobilised rat testes. Magnification, x40

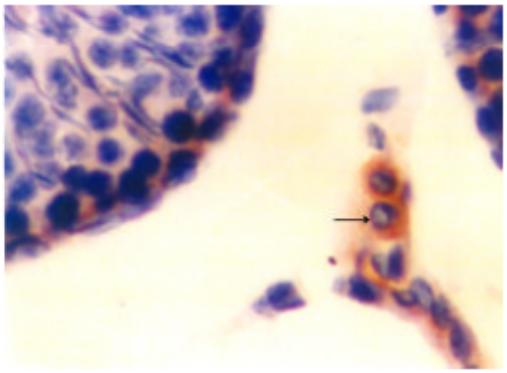

Fig. 3: Immunohistochemical staining of Bax observed at strong level (+++) in the Leydig cells (→) of immobilised rat testes. Magnification, x40

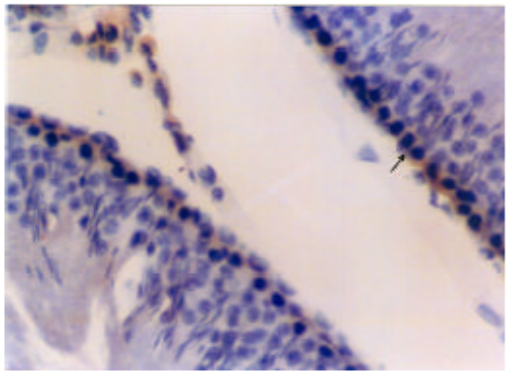

Fig. 4: Bcl-2 immunoreactivity observed at peak level (+) in the spermatogonia (→) of the immobilised rat testes. Magnification, x20

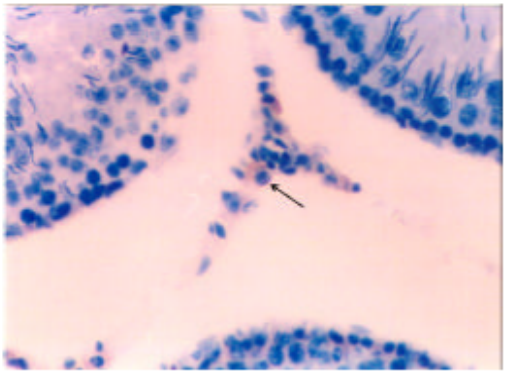

Fig. 5: Decreased Bax immunoreactivity (+) observed in Leydig cells (→) of vitamin E treated rats during immobilisation. Magnification, x20

(Fig. 3) of immobilised rats. In this group, Bcl-2 protein was expressed at peak level (+) in the seminiferous tubules (Fig. 4) and similar to control group. In the vitamin E treated group, Bax and Bcl-2 expressions in spermatogonia and intersitisyal cells were decreased and peak level (+) (Fig. 5).

DISCUSSION

Apoptosis in the testes is an important physiological mechanism that regulates the number of germ cells in the seminiferous epithelium.

Bcl-2 family proteins mainly play a role in the spontaneous apoptosis during normal spermatogenesis. Bcl-2 and Bak proteins are expressed in the spermatocytes and differentiating spermatids, Bax expression is in spermatids with round nuclei (Oldereid *et al*., 2001).

Stress is believed to influence male reproductive activity and increases the apoptotic index in the seminiferous tubules of the rat testes (Yazawa *et al*., 1999). Apoptotic germ cells, most frequently spermatogonia and primary spermatocytes, were detected in the testes of immobilised rats for 2 h daily on 7 consecutive days (Yazawa *et al*., 1999). It was reported that testes weight was normal but spermatid production decrease in the rats after 6 h of immobilisation for 15 days (Almeida *et al*., 2000). Testes weights and spermatogenesis were significantly decreased and germ cell loss occurred in the seminiferous tubules in the long-term hindlimb suspension in adult male rats. Also, they observed that Leydig and Sertoli cells showed normal structure in the same experimental groups (Tash *et al*., 2002).

In this study was observed that body weights of both immobilised and vitamin E treated groups significantly decreased. It can be associated with stress dependent anorexia. Testes weights of all groups unchanged which is paralleled to result of Almeida *et al*. (2000). They have reported that testes weight was normal but spermatid production decreased in the rats after 6 h of immobilization for 15 days. Bax (+++) expression has been detected in the spermatogonia in some seminiferous tubules of the immobilised rats. Bcl-2 (+) expression was observed less than Bax expression in the spermatogonia. These data suggests that apoptosis-related Bax protein plays a role in the immobilisation stress-induced spermatogonia apoptosis. These results are similar to that of Russell *et al*. (2002) demonstrated that Bax protein played role in the especially spermatogonia apoptosis. An other result in the present study is strong Bax expression observed in the Leydig cells of the immobilised rats. Recent studies suggested that Bcl-2 inhibited apoptosis in the Leydig cells, but Bax, Fas and Caspase-3 stimulated apoptosis (Yuan and Xu, 2003). Alcohol induces apoptosis by increasing Bax expression in Leydig cells. On the other hand it decreases Bcl-2 expression (Jang *et al*., 2002).

Leydig cells may have undergone apoptosis due to decrease in serum hormone levels during immobilization stress as described in previous studies (Demura *et al*., 1989). We observed that Bax and Bcl-2 expression were not present in seminiferous tubules of vitamin E treated groups. It was reported that vitamin E prevented neuronal cells-apoptosis (Choi *et al*., 2003). Lung phospholipid, unsaturated lipids were decreased in the immobilisation stress and vitamin E inhibited this changes (Kovacheva and Ribarov, 1995).

CONCLUSION

This study has demonstrated that vitamin E treatment has effect on the immobilisation stress-induced apoptosis and decrease especially Bax expression in spermatogonia.

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