Effect of oxidative stress on the spermatogenic process and hsp70 expressions in mice testes

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The effect of oxidative stress on the process of spermatogenesis in terms of hsp70 expression was studied. For creating different oxidative stressed mice, three selenium (Se) levels viz., deficient (group I), adequate (group II) and excess (group III) were fed for 8 weeks in a yeast-based diet. After completion of diet feeding, Se level was significantly decreased in group I and significantly increased in group III, as compared to group II. Glutathione peroxidase (GSH-Px) activity was significantly decreased in both liver and testis in group I animals; however, the activity was comparable in groups II and III. Significant increase in the testis glutathione-S-transferase (GST) activity was observed in group I. No change was seen in group III, when compared to group II. Histological analysis of testis revealed a significant decrease in the germ cell population in group I, as compared to group II, with a predominant effect on spermatid and mature sperm numbers. In group III, displacement of germ cell population was observed. ELISA assays for hsp70 level showed increase in group I as compared to group II, whereas no significant change was observed in group III, as compared to group II. Immuno-histochemical analysis revealed intense localization of hsp70 only in spermatid and sperm cells. The expression in groups II and III was homogeneous with slightly increased expression around lumen in group III. The data indicate that excessive oxidative stress in Se deficient group, affects the spermatogenesis process, especially affecting the mature sperm number which in turn leads to infertility.

Key words: selenium, testis, hsp70, oxidative stress

Endogenous oxygen radicals generated as by-products of cellular metabolism result into DNA damage, protein inactivation and cell membrane instability1. Oxidative damage in human spermatozoa is associated with loss of motility, decreased fusion capability, DNA damage and higher frequency of abnormal sperm with significant effect on male fertility2,3. Extremely high concentrations of polyunsaturated fatty acids (PUFA), along with their ability to generate reactive oxygen species (ROS) render human spermatozoa particularly susceptible to peroxidative damage4,5. A number of antioxidant systems play a role to counteract the effect of ROS6. Selenium (Se) is shown to be essential for male fertility7. The biological functions of Se in mammals appear to be expressed through different biologically active compounds, including glutathione peroxidase (GSH-Px)8. The protective function of Se from damage by free radicals may be related to its antioxidant property. The toxicity of lipid peroxidation products in the cell is reduced, in part by glutathione-S-transferase (GST). Multiple isoforms of GST are present in the testis and are considered to protect germ cells from electrophilic compounds and reactive oxygen intermediates, which are potentially hazardous to integrity of DNA and may have profound effect on sperm9,10. The contribution of GST in detoxifying the product of oxidative damage becomes quite significant in situation of Se deficiency, where Se-dependent GSH-Px and PHGPx are reduced11.

To maintain the homeostasis under oxidative stress, the testis germ cells produce high levels of stress proteins or hsps, which protect against the damage12. Environmental and physiological stress have been shown to induce the expression of hsp7013 (the most frequently studied hsp). Members of hsp70 protein family act as chaperone, which assist in the folding, transport and assembly of protein in cytoplasm, mitochondria and endoplasmic reticulum14. The present study was aimed to study the influence of oxidative stress levels as created in different Se (a potent anti-oxidant) status mice on spermatogenic cells and immuno-histochemical localization of hsp70 proteins in relation to reproductive activity.
Materials and Methods

Male Balb/c mice (weighing 25 g) were procured from Central Animal House, Panjab University, Chandigarh. To create different oxidative conditions in mice, three different Se status animal groups viz., 0.02 ppm (group I), 0.2 ppm (group II) and 1 ppm (group III) were created. Mice were kept on yeast-based diet, which usually contains 0.02 ppm Se, and thus animals fed on this diet for 8 weeks were considered Se-deficient. Animals in groups II and III were fed Se deficient diet supplemented with sodium selenite at 0.2 ppm (adequate) and 1 ppm Se level (excess levels). All the animals were fed with the respective diet for 8 weeks and allowed water ad libitum.

Selenium concentration in the testis was determined by flourimetric method. Glutathione peroxidase (GSH-Px) activity was measured in the liver and testis post mitochondrial fraction (PMF) as described, while glutathione-S-transferase activity was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) as the substrate.

Hsp70 analysis

Hsp70 analysis in testis from all the three experimental groups was done using Western immunoblot and Elisa assay. SDS-PAGE (10% gel) of testis cytosol (10 μg protein) was carried out and blotted onto polyvinylidene difluoride (PVDF) membrane. Immunoblot was prepared using monoclonal anti hsp70 antibody as primary antibody and biotin labeled antimouse IgG as secondary antibody. Peroxidase and DAB plus H2O2 detection system was used.

Enzyme linked immunosorbent assay was used for hsp70 levels. Microtitre plate was coated with 10 μg protein in 100 μl of carbonate buffer (0.05 M, pH 9.6) overnight at 4°C. Wells were then blocked with 100 μl PBS containing 1% BSA for 1 hr at 37°C and washed with 200 μl PBS containing 0.05% (v/v) tween-20. The 100 μl of primary monoclonal anti hsp70 antibody (diluted 1:1000 in PBS containing 0.05% tween-20 and 1% BSA) was added to each well and kept for 2 hr at 37°C. Plate was again washed with PBS containing 0.05% (v/v) tween-20 and incubated with biotin-labeled antimouse IgG as secondary antibody, followed by streptavidin-labeled peroxidase. The colour was developed by adding 2,2’-azino-di-(3-ethyl-benezo-thiozolin sulfonic acid) reagent.

Histopathological studies

Fresh pieces of testes were taken randomly immediately after sacrifice of the animals from the three experimental groups, fixed in zenker fixative for 24 hr, processed with different grades of alcohol, embedded in paraffin wax, sectioned (8 μ thick) and stained with haemotoxylin/eosin.

Immuno-histochemical analysis for hsp70

Deparaffinized sections were incubated with 3% H2O2 at 37°C, to block the endogenous peroxidase activity. Sections were then blocked with blocking solution (2% BSA in PBS) for 30 min at 37°C and incubated with monoclonal anti hsp70 antibody (diluted 1:2000) in PBS containing 1% BSA. For negative control, only diluent (PBS with 1% BSA) was added. Slides were dipped in 0.05% (v/v) tween-20 in PBS and incubated with biotinylated antimouse IgG (suspended in PBS containing 1% BSA/PBS) for 45 min. Sections were then incubated with avidin-biotin peroxidase

<table>
<thead>
<tr>
<th>Testis Se status (μg/g tissue)</th>
<th>GSH-Px activity (μmol NADPH oxidized/min/mg protein)</th>
<th>Testis GST activity (μmoles of CDNB conjugated/min/mg protein)</th>
<th>Testis hsp70 level (A405)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Testis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I 0.53 ± 0.04**</td>
<td>283.76 ±14.0**</td>
<td>97.91±3.7</td>
<td>27±1.1**</td>
</tr>
<tr>
<td>Group II 0.91± 0.04</td>
<td>910.12±21.4</td>
<td>220.86±10.3</td>
<td>16.3±1.0</td>
</tr>
<tr>
<td>Group III 1.22± 0.03*</td>
<td>933±16.1</td>
<td>227.71±9.2</td>
<td>17.5±2.1</td>
</tr>
</tbody>
</table>

**p<0.001 (comparison with group II); *p<0.01 (comparison with group II)
complex for 45 min. The reaction product was developed using DAB plus H$_2$O$_2$. Sections were mounted in glycerol jelly.

**Results**

*Selenium status*

The testis Se was found to be significantly low in animals fed on Se deficient diet (group I), as compared to group II after 8 weeks of respective diet feeding (Table 1). In animals fed on 1 ppm Se diet (group III), testis Se level was found to be significantly high as compared to adequate Se diet fed group.

*GSH-Px and GST activities*

Table 1 shows GSH-Px activity in testis and liver of animals from the three groups. A significant decrease in the enzyme activity was observed both in liver and testis of group I animals, as compared to group II. However, no significant change in the enzyme activity was observed in group III, as compared to group II. A significant increase in the GST activity was observed in testis of group I animals. In group III, the enzyme activity was found to be comparable to group II (Table 1).

*Histopathological analysis*

Photomicrograph of transverse section of testis from the three groups (Fig. 1 A-C) shows shrinkages of seminiferous tubules in group I (Fig. 1A), as compared to group II (Fig. 1B). Decrease in the germinal height and increase in central lumen size was also observed in group I (Fig. 1A), as compared to group II. Various stages of meiosis were not clearly visible and there was an appreciable decrease in the spermatid and mature sperm number in group I. In group III, shrinkage of tubules was quite apparent (Fig. 1C). The central lumen decreased in size and the displacement of germ cell population was quite frequent. However no appreciable decrease was seen in the spermatid and mature sperm, as compared to the adequate group.

*Hsp70 analysis*

The Western immunoblot for the hsp70 in the three different oxidative stress animals showed a single band at 70 kDa in all the samples on Western transfer of SDS-PAGE (Fig. 2).

Hsp70 was quantitated in testis cytosol of all the three experimental groups using ELISA. Table 1 shows the absorbance (A$_{405}$) in wells on ELISA reaction, which is taken as a direct assay of hsp70 concentration. No significant change was observed in the hsp70 level in the testis sample from group III animals. On the other hand, hsp70 level was found to be increased in group I, as compared to the group II.

*Immuno-histochemical analysis for Hsp70*

Fig. 3 A-C shows the photomicrograph of testis sections from all the three groups. The negative
control (without antibody against hsp70) did not show any detectable staining. Hsp70 expression in group I animals was found to be very intense in spermatid and sperm head region (Fig. 3A), while the various stages of meiosis including primary spermatocyte seem to have a lower expression than group II mice. Group II showed a homogenous distribution of hsp70 in the seminiferous tubule (Fig. 3B). The hsp70 expression in group III animals was comparable to group II with slightly increased expression around the lumen of the seminiferous tubule (Fig. 3C).

Discussion

Reactive oxygen species (ROS) are involved in the peroxidative damage of human spermatozoa, which may result in male infertility. The high rate of mitosis and various stages of meiosis in the seminiferous tubule expose the germ cell chromosome to potentially damaging influences of free radicals in local environment, thus creating a need for an effective antioxidant system. Selenium, an essential trace element for higher eukaryotes and many bacteria, is essential in the activity of antioxidant enzymes glutathione peroxidase (GSH-Px) and phospholipid hydroperoxide glutathione peroxidase (PLGSH-Px). GSH-Px, a constituent of the cellular antioxidant defense system is located in cytoplasm and mitochondria. Se is an integral cofactor of GSH-Px, which removes hydrogen peroxide and lipid peroxides and thus protects spermatozoa from peroxidative damage. Glutathione and glutathione dependent enzymes represent a coordinately regulated defense against oxidative stress, suggesting that Se level plays a role in regulating the level of oxidative stress. Studies have reported that GST directly take part in the elimination of the products of peroxidation and there is enhancement of GST activity in germ cells after exposure to H₂O₂. Thus, increased GST activity observed in group I (Table 1) suggests increased oxidative stress in the testis.

The histological changes in the testis in groups I and III were observed, as compared to group II. There was a marked decrease in the spermatid and mature...
sperm in group I (Fig. 1A). Decrease in the pachytene spermatocytes was also observed. This decrease could be due to oxidative stress. H$_2$O$_2$ and lipid peroxides are shown to be toxic for spermatozoa$^{25}$. The decrease in the GSH-Px level in group I (Table 1) also results in oxidative stress, since it is known to protect cell against damage caused by free radicals and the product of lipid peroxidation in vivo$^{26}$. Significant increase in the GST enzyme activity also suggests increased oxidative stress$^{11}$. Marked alteration in the physico-chemical state of the DNA protein complex in spermatozoa chromatin has been observed by various researchers during spontaneous oxidation$^{27}$. Group III shows the displacement of germ cell population (Fig. 1C). This small damage may be attributed to the ability of all Se compounds at supranutritional level to generate oxidative stress through production of selenopersulfide within cell$^{28}$.

In situation of stress exposure due to oxygen radicals or cytokines$^{29}$, the constitutive cellular pool of hsps become inadequate and synthesis of hsps is readily induced to increase cellular defense. Hsps act as molecular chaperones$^{30}$ by helping in the correct folding and unfolding of nascent polypeptides to achieve a functional conformation$^{30}$, assist the translocation and/or delivery of newly synthesized proteins to the proper intracellular target or along secretory pathways$^{31}$, interact with proteins of the cytoskeleton$^{32}$ and modulate binding of steroid hormones to their receptor$^{33}$. The inducible form of hsp70 (ihsp70) is shown to be cytoprotective in a number of studies using various models and different stresses$^{34,35}$. High amounts of hsps are produced by the testis germ cells to provide protection against damage and maintain homeostasis under the stress condition$^{12}$.

In addition to the inducible form of hsp70, spermatogenic cells also express the constitutive form of hsp70 known as hsp70-2$^{36}$. The synthesis of hsp70-2 occurs in the meiotic phase of spermatogenesis and is abundant in pachytene spermatocyte. It is required for desynapsis of synaptonemal complex during meiotic phase in juvenile and adult mouse spermatocyte$^{37}$. In the present study, hsp70 level was found to be significantly increased in group I; however, the increase was not significant in group III, as compared to group II. This increase in hsp70 level could be due to the excess oxidative stress in group I animals. However, as there is 80% homology between hsp70 and hsp70-2, the presently used antibody may also be recognizing hsp70-2. Thus, to examine the exact status of hsp70-2, analysis need to be done with more specific antibody.

Immuno-histochemical analysis for hsp70 in group I (Fig. 3A) showed very intense staining in the spermatid and sperm head region of seminiferous tubule. Morphological studies have indicated that round spermatid are the most sensitive in condition of heat stress$^{38}$. The greater susceptibility of spermatids to stress has also been reported recently$^{39}$, which could also explain for increased hsp70 expression in the spermatid region under the oxidative stress. Further, in terminal stages of sperm differentiation, since the considerable portion of the sperm cytoplasm is discarded$^{19}$, as a consequence these cells are not well endowed with cytoplasmic defense system that protect cell from peroxidative damage. This accounts for increased hsp70 expression in sperm head region under Se deficient condition. The hsp70 expression in various other stages is very less as compared to group II (Fig. 3B) which can be the result of interruption of meiotic process in group I and hence decrease in the hsp70-2 expression which appears abundantly during meiosis. The hsp70 expression in group III (Fig. 3C) is comparable to that of group II, except for slightly increased expression surrounding the lumen, which may be due to mild oxidative stress in group III due to the production of selenopersulfide within the cell.

In conclusion, the experimental oxidative stress generated in the present study clearly demonstrates the reduction in the spermatogenic activity, which will have a direct influence on fertility. Secondary hsp70 expression in particular cells also demonstrates the system reactivation against oxidative damage.

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