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ORIGINAL CONTRIBUTION

Selenium variation induced oxidative stress regulates p53 dependent germ cell apoptosis: plausible involvement of HSP70-2

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Abstract
Background Selenium at altered concentration causes abnormal spermatogenesis and male infertility. However, the exact mechanism behind this is still unexplored.
Aims It was aimed to investigate if Se induced oxidative stress alters the expressions of testis specific HSP70-2 protein, that is crucial in normal spermatogenesis. The study was extended to delineate the apoptotic process after this change if any.
Methods To create different Se status-deficient, adequate and excess, male Balb/c mice were fed yeast based Se deficient diet (group I) and this diet supplemented with Se as sodium selenite at 0.2 and 1 ppm Se (group II and III, respectively) for 8 weeks.
Results After the feeding schedule, a dose dependent change in the Se levels and GSH-Px activity was observed in samples of different Se diet fed group animals as reported in earlier studies. Changes in the redox status in both groups I and III indicated oxidative stress conditions. The mRNA and protein expression of HSP70-2 was found to be reduced in group I and III, whereas, the expressions of p53 demonstrated a reverse trend. Increased apoptosis was observed in the group I and III animals as indicated by increased apoptotic index (AI) on the TUNEL stained sections and by DNA fragmentation indicating DNA damage in these groups.
Conclusion These findings suggest that Se variations induced oxidative stress leads to germ cell apoptosis and downregulation of HSP70-2. This study suggests that there can be a possible link between these two events and the fate of HSP70-2 in case of oxidative damage can provide an insight into the mechanism(s) by which the nutritional variation induced oxidative stress can affect reproductive potential and thus demonstrates the importance of nutrition at molecular level as well.

Keywords Selenium · Oxidative stress · HSP70-2 · p53 · Apoptosis, apoptotic index

Abbreviations

Se	Selenium
HSP	Heat shock protein
TUNEL	Terminal dUTP linked nick end Labelling
MDA	Malondialdehyde
RT-PCR	Reverse transcription polymerase chain reaction
GSH-Px	Glutathione peroxidase
ROS	Reactive oxygen species
AI	Apoptotic index

Introduction

The biological Se research can be broadly divided into two distinct areas, which involve the study of Selenium's nutritional essentiality and its toxicity. Selenium (Se), an essential trace element is of fundamental importance in testicular functions and male fertility at physiological concentrations [4]. Any deficiency in the nutritional and physiological selenium levels adversely affects testicular mass, morphology and function. In livestock and experimental rodents, selenium deficiency leads to alteration or complete abrogation of spermatogenesis [4]. Previous evidences support the fact that the reduction in

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Se concentration theoretically renders spermatozoa more vulnerable to oxygen radicals, therefore, linking low Se status with increased expression of genes involved in oxidative stress [25].

On the contrary, the excessive supplementation of Se is toxic and affects the male fertility [30] by causing increased incidence of midpiece abnormalities in sperms [18]. Also, at high concentration, selenite is known to induce apoptosis in cells via a free radical mechanism [28]. The fact that Se is incorporated during the spermatocyte or early spermatid stage leads to the possibility that this element can regulate the spermatogenic process during the initial stages of spermatogenesis.

However, the literature explaining the exact nature of defects associated with excess selenium supplementation is still scarce. Furthermore, the effects of selenium deficiency on spermatogenesis may have been grossly studied, but the phenomena/mechanisms behind these have not yet been explored [13].

HSP70-2 a testes specific gene is expressed in spermatogenic cells during the prophase of meiosis I in male germ cells [33]. Its expression during initial stages of spermatogenesis reveals that this protein has an essential chaperone role during the G2/M phase transition of the meiotic phase of spermatogenesis where it is required for cyclin dependent kinase (Cdc2) activation and Cdc2-cyclin B1 assembly [10, 34]. It has been observed that gene disruption of HSP70-2 or mutations in this gene lead to decreased testicular weight and infertility caused due to apoptosis of late pachytene spermatocytes [8]. Also, the fragmentation of DNA which is consistent with interchromosomal chromatin degradation typical of apoptosis has also been observed in HSP70-2$^{-/-}$ animals [8, 24].

Considering this background in mind, the current study was designed to study the changes in the expression of HSP70-2 expression following oxidative stress caused by variations in the dietary selenium concentrations. Also, the possibility of apoptosis and DNA damage in germ cells was explored after free radical mediated changes in the HSP70-2 expression. Finally, the study was extended to delineate the plausible role of p53 in the apoptotic pathway stemming from the modulation of HSP70-2 as caused by differential selenium status mediated oxidative stress in the germ cells.

Materials and methods

Chemicals

Sodium selenite (Na_2SeO_3) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TRI-reagent and one-step RT-PCR kit were obtained from Molecular Research Centre (Inc. Cincinnati; Ohio) and QIAGEN, respectively. Oligonucleotides were synthesized by Sigma-Aldrich. PVDF membrane was purchased from Millipore (USA). The primary antibodies for p53, caspase 3, caspase 9, β actin and peroxidase conjugated secondary antibodies were from Sigma-Aldrich, respectively, whereas the antibodies for HSP70-2 were a kind gift from Dr. E. M. Eddy (Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NIH, USA).

Animals and experimental design

Male Balb/c mice of approximately 25 g body weight were procured from the Central Animal House, Panjab University, Chandigarh (India). All the experiments and protocols were approved by the institutional animal ethical committee. Animals were acclimatized to the departmental animal rooms for a period of 1 week prior to the start of the treatment. The animals were randomly segregated in three groups to create different oxidative conditions by different Se status viz. 0.02 ppm (group I), 0.2 ppm (group II) and 1 ppm (group III) using special yeast based diet for a period of 8 weeks.

Diet preparation

Yeast based synthetic Se deficient diet (contain 0.02 ppm Se) was prepared in the laboratory itself according to the composition given by Burk [5] as described by us earlier [19]. Se adequate and excess diets were prepared from Se deficient diet by supplementing it with 0.2 and 1 ppm of Se, respectively, as sodium selenite. After completion of diet feeding schedule of 8 weeks, animals were sacrificed under barbitone intoxication; testes were removed and used for various analyses.

The testes and liver from different treatment groups were analyzed for Se levels. Different Se concentration supplemented diets have been reported to create differential Se status in respective groups and concomitant changes in the Se-dependent GSH-Px activity by respective methods as described by us earlier [19].

Estimation of glutathione levels

The post mitochondrial fraction (PMF) was obtained from tissue homogenates (10% w/v) and the levels of total and oxidized glutathione were measured by the fluorimetric method of Hissin and Hilf [14]. This method is based on the principle that glutathione reacts specifically with *o*-pthaldehyde (OPT) at pH 8 and with GSSG at pH 12 resulting in the formation of a highly fluorescent product which is activated at 350 nm with an emission at 420 nm.

RNA isolation and mRNA expression of HSP70-2 and p53

Total RNA from testis was extracted using TRI-reagent. The integrity and size distribution (quality) of RNA was examined on 1.2% formaldehyde agarose gel electrophoresis.

The mRNA expression of HSP70-2 and p53 were analyzed in testis using RT-PCR kit from QIAGEN as described earlier [20]. The annealing temperatures used for RT-PCR was 54°C for HSP70-2, p53 and β actin (internal control). The optimal primer sequences used for analyses of various genes are as shown below in Table 1. Final PCR products formed were analyzed on 1.5% agarose gel electrophoresis and densitometric analysis of the bands was done by Image J software (NIH, USA). Mean of four independent densitometric analyses of PCR product bands were determined for comparison of each analysis.

Western immunoblot analysis

Protein samples (40 μg) from each treatment group were separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to the PVDF membrane. Different immunoblots were prepared using respective antibodies (antisera against mouse HSP70-2, anti-mouse anti-p53 (primary antibodies) and further by peroxidase conjugated anti-rabbit IgG as secondary antibody. Diaminobenzedine (DAB) plus H_2O_2 detection system was used to develop the blot. Mouse β actin was used as internal control.

Apoptotic studies

The occurrence of apoptosis in the testicular germ cells of various treatment groups was studied using DNA ladder studies and then correlated with frequency of various germ cells to undergo apoptosis by measuring apoptotic index on sections prepared for TUNEL analysis.

Table 1 Primer pairs used

Gene	Primer
HSP70-2	
Sense	5′-AGA CGC AGA CCT TCA CTA C-3′
Antisense	5′-TTT TGT CCT GCT CGC TAA TC-3′
p53	
Sense	5′-GGG ACA GCC AAG TCT GTT ATG-3′
Antisense	5′-GGA GTC TTC CAG TGT GAT GAT-3′
β actin	
Sense	5′-ATC CGT AAA GAC CTC TAT GC-3′
Antisense	5′-AAC GCA GCT CAG TAA CAG TC-3′

DNA fragmentation analysis

The total genomic DNA from all the three treatment groups was separated using standard chloroform:phenol method and subjected to agarose gel electrophoresis using 1.8% agarose gel.

Quantification of apoptotic cells

The percentage of apoptotic cells was determined by calculating the apoptotic index. The apoptotic index (AI) was calculated in each section by dividing the number of apoptosis positive germ cells per seminiferous tubule by the total number of total germ cells per seminiferous tubule (obtained from H and E slides) and the result was multiplied by 100. Mean AI of each case was calculated. The data was then analyzed using student's *t* test.

Statistical analysis

Data is expressed as Mean ± SD of at least six independent observations for all biochemical estimations. Differences between different groups was tested using student's *t* test for unpaired values. RT-PCR and western immunoblot densitometric analysis were carried from four independent observations using Image J Software (NIH, USA).

Results

Estimation of glutathione levels

The levels of oxidized (GSSG) and reduced (GSH) glutathione were estimated in testis after 8 weeks in three treatment groups (Table 2). A statistically significant increase was observed in GSSG in group I and III when compared to group II animals. A highly significant decrease in GSH levels was also demonstrated in both Se deficient (group I) and Se excess (group III) fed groups in comparison to the animals fed on adequate selenium supplemented diet (group II). Significant decrease in the redox ratio (GSH/GSSG) was observed in group I and group III as compared to Se adequate fed group II animals.

mRNA and protein expression of HSP70-2 and p53

A significant decrease in the mRNA and protein expression of HSP70-2 was observed under the conditions of Se deficiency and excess as compared to the Se adequate. However, this decrease was more prominent in the Se deficient group (Figs. 1, 2). On the contrary a statistically significant increase in the expression of p53 mRNA and

Table 2 Glutathione levels and apoptotic index in mice testis

Se deficient Group I	Se adequate Group II	Se excess Group III
Reduced glutathione (GSH) (nmoles/mg protein)		
15.15 ± 1.33***	30.90 ± 0.80	19.92 ± 1.54***
Oxidized glutathione (GSSG) (nmoles/mg protein)		
2.23 ± 0.38***	1.67 ± 0.14	2.08 ± 0.07**
Redox ratio (nmoles/mg protein)		
6.86 ± 0.72***	18.50 ± 1.49	9.56 ± 0.61***
Apoptotic index		
23.06 ± 4.02***	9.86 ± 2.34	18.62 ± 2.96***

The values are mean ± SD of six independent observations

,* $p < 0.01$, $p < 0.001$, respectively

protein was observed (Figs. 1, 2) in the animals fed Se deficient (group I) and Se excess (group III) supplemented diet as compared to Se adequate fed group (group II).

Apoptotic studies

By TUNEL analysis it has been found in the earlier studies that the frequency of apoptotic germ cells was more in the testicular sections of Se deficient and Se excess animals as compared to the Se adequate fed group.

The percentage of apoptosis was measured in these TUNEL stained as apoptotic index (AI). In Se adequate group very low numbers of apoptotic germ cells were observed. However, the mean apoptotic index (AI) in Se deficient and excess diet fed groups was significantly higher than in adequate Se fed animals (Table 2).

DNA ladder studies

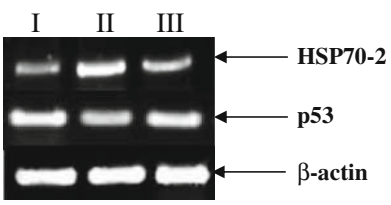
A major single band of very high molecular weight was observed in all the three preparations of DNA from three groups (Fig. 3). However, DNA sample from group I animals (lane I) and from group III animals (lane III) also showed low molecular weight fragments. These fragments confirm the fact that in the conditions of selenium deficiency and selenium excess, the DNA damage occurs.

Discussion

In earlier reports, we have already demonstrated the establishment of differential Se status in the animals fed on diet containing different concentrations of Se. This fact has also been confirmed by concentration dependent changes in the Se-dependent GSH-Px activity in these studies [19].

The decreased GSH levels in Se deficient and excess group demonstrates increased utilization of GSH in the removal of free radicals and peroxides. Simultaneously, the levels of GSSG were found to increase. These changes in the levels of these peptides may be due to a reduced expression of γ glutamylcysteine synthetase (γGCS), which is rate limiting enzyme in glutathione metabolism. Fischer et al. [12] demonstrated that Se deficient diet feeding reduced the γGCS expression in rat liver. The reduced GSH levels in the animals supplemented a diet with excess selenium may be due to the ability of excess selenium to react with GSH in a redox cycling pathway, building up free radicals in the system and diminishes the GSH pool [28, 31].

A testes specific constitutive form of HSP70 known as HSP70-2 is expressed in spermatogenic cells during the prophase of meiosis I in male germ cells [34]. The mRNA and protein expression of HSP70-2 was found to be reduced under Se deficient and excess conditions compared to adequate Se fed group. We have already reported a decreased expression and kinase activity of Cdc2 [20], which can be due to this reduced expression of HSP70-2, as



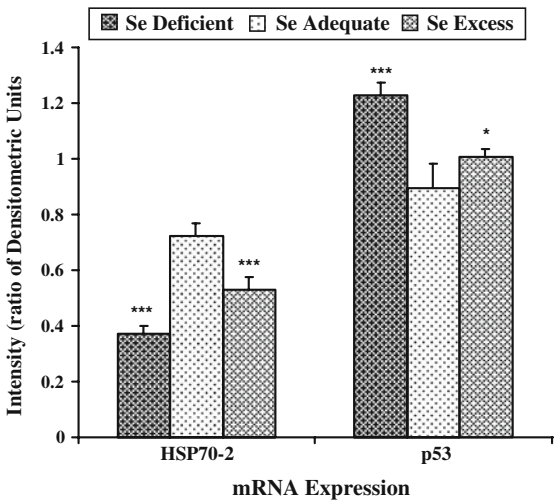


Fig. 1 Effect of selenium induced oxidative stress on the mRNA expression of HSP70-2 and p53 (**a**) and their densitometric analysis (**b**). *Lane I* Se deficient, *lane II* Se adequate, *lane III* Se excess. The values are mean ± SD of four independent observations. *,*** $p < 0.05$, $p < 0.001$, respectively

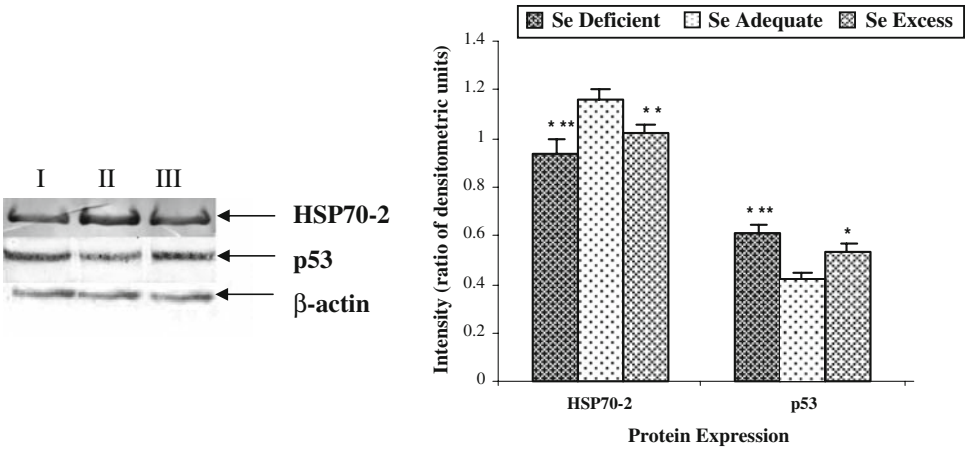


Fig. 2 Effect of selenium induced oxidative stress on the protein expression of HSP70-2 and p53 (**a**) and their densitometric analysis (**b**). *Lane I* Se deficient, *lane II* Se adequate, *lane III* Se excess. The values are mean ± SD of four independent observations. *,**,*** $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively

this protein is a chaperone required for cyclin dependent kinase (Cdc2) activation and Cdc2-cyclin B1 assembly [10, 34]. These findings are supported by the fact that the human HspA2 gene, which is a human homologue of mouse HSP70-2 gene [15] and having 98.2% amino acid sequence identity is also repressed in testes with abnormal spermatogenesis [29].

Although ample evidences point towards the role of HSP70-2 in male infertility but still the exact role of this protein in meiosis is not known [7]. However, reports exist which suggest that the fragmentation of DNA, which is consistent with inter-chromosomal chromatin degradation typical of apoptosis, has also been observed in HSP70-2$^{-/-}$ animals [7, 8].

DNA fragmentation in testis from group I and group III animals may be attributed to the enhanced oxidative stress. Recently, oxidative stress has emerged as potential candidate that plays a pivotal role in the induction of apoptotic cell death [9]. Kumar et al. [22] have also demonstrated that increased lipid peroxidation is concurrent with the oxidative damage to testicular DNA. Furthermore, it has been reported that infertile men with poor sperm motility and morphology have increased DNA fragmentation compared with individuals with normal semen parameters [17, 35]. These observations support our study that reports diminished reproductive parameters with DNA fragmentation in selenium deficient and excess group animals. At supranutritional levels, selenium compounds form selenols, which are catalytic [28] and may further cause DNA damage [21].

Apoptotic index was measured to calculate the changes in the number and susceptibility of germ cells to undergo apoptosis under different nutritional Se levels. Elevated levels of apoptosis are more prevalent in the Se group I and Se excess group III. This might be due to the deregulation in spermatogenesis and DNA repair leading to arrest at this stage of spermatogenesis [11]. The increased apoptosis in these groups could also be due to faulty meiotic recombination's in spermatocytes, disruption of the meiotic cell

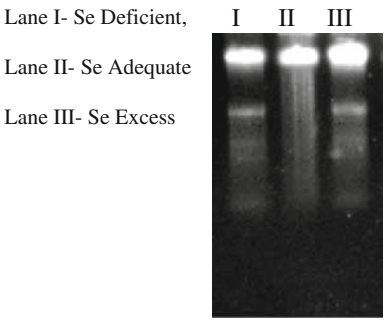


Fig. 3 Agarose gel electrophoresis of genomic DNA extracted from mice testis from various treatment groups. (10 μg DNA/lane). *Lane I* Se deficient, *lane II* Se adequate, *lane III* Se excess

cycle regulatory machinery [34] or direct perturbation of apoptosis regulations in spermatocytes. These observations are in collaboration with the decreased expression of HSP70-2 in these groups, which has been found to modulate the process of apoptosis in spermatocytes, spermatids and even ejaculated mature sperms [3].

p53, a sensor molecule is considered a prime inducer of apoptosis within germ cells, as it is present in unusually high concentrations particularly in pachytene spermatocytes which represents the cell stage to be the most venerable to stress [1, 26]. Also, there is an implication of p53 in the G2 checkpoint but the molecular mechanisms involved are less clear. Furthermore, the relationship between selenium and p53 has been observed recently [23, 27].

In the present study, a highly increased expression of p53 was observed in both selenium deficient and selenium excess diet fed groups as compared to the adequate diet fed animals. In the conditions of selenium deficiency induced oxidative stress, the induced expression of p53 might be due to increased stabilization and half-life of p53 as suggested earlier in response to DNA damage and oxidative stress [6]. In the excess conditions, the enhanced expression of p53 can be explained on the basis of activation of a

p53-dependent repair mechanism. Supplementation of cells with selenium or selenomethionine is found to activate p53 [27].

The increase in the expression of p53 explains the possibility of cell cycle arrest at G2/M phase checkpoint following oxidative stress mediated DNA damage where HSP70-2 has the most critical role. The picture that emerges point to the direction that decreased HSP70-2 can be linked to increased p53 expression and can lead to trans-repression of Cdc2 and cyclin B1 by interacting with their promoter [16, 32]. This hypothesis is supported by the fact that down-regulation of both p34cdc2 and cyclin B1 and a concomitant decrease of p34cdc2 activity is attributed to a functional p53 [2].

In conclusion, the above findings demonstrate that Se variation induced oxidative stress mediates germ cell apoptosis in a p53 dependent apoptotic process that can lead to male infertility. The downregulation of HSP70-2 as seen in current study under Se deficient and excess conditions may have an important role in germ cell apoptosis. Also, there is a possibility of involvement of multiple pathways regulating the germ cell apoptosis operating at different molecular level/s and the changes in expression of related genes consisting of a cascade in apoptosis might be meaningful for the modulation of apoptotic processes and design of superior treatment modalities for male infertility.

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