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Dietary selenium variation-induced oxidative stress modulates CDC2/cyclin B1 expression and apoptosis of germ cells in mice testis

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Abstract

Oxidative stress has been linked with apoptosis in germ cells and with male infertility. However, the molecular mechanism of oxidative-stress-mediated apoptosis in germ cells has not been clearly defined so far. Because of the involvement of CDC2 and cyclin B1 in cell cycle regulation and their plausible role in apoptosis, the present study aimed to investigate the possibility that selenium (Se)-induced oxidative-stress-mediated modulations of these cell cycle regulators cause DNA damage and apoptosis in germ cells. To create different Se status (deficient, adequate and excess), male Balb/c mice were fed yeast-based Se-deficient diet (Group I) and a deficient diet supplemented with Se as sodium selenite (0.2 and 1 ppm Se in Groups II and III, respectively) for a period of 8 weeks. After the completion of the diet feeding schedule, a significant decrease in Se levels and glutathione peroxidase activity was observed in the Se-deficient group (Group I), whereas the Se-excess group (Group III) demonstrated an increase in Se levels. Increased levels of lipid peroxidation were seen in both Groups I and III when compared to Group II, indicating oxidative stress. The mRNA and protein expressions of both CDC2 and cyclin B1 were found to be significantly decreased in Groups I and III. A decrease in the immunohistochemical localization of these proteins was also observed in spermatogenic cells. The mRNA expressions of apoptotic factors such as Bcl-2, Bax, caspase-3 and caspase-9 were found to be increased in Groups I and III. A decrease in CDC2 kinase activity was also seen in these groups. Increased apoptosis was observed in Group I and Group III animals by terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling assay indicating oxidative-stress-mediated DNA damage. These findings suggest the effect of Se-induced oxidative stress on the cell cycle regulators and apoptotic activity of germ cells, thus providing new dimensions to molecular mechanisms underlying male infertility.

Keywords: Selenium; Oxidative stress; CDC2; Cyclin B1; Apoptosis

1. Introduction

Spermatogenesis is a paradigm of development that is orchestrated by a specific genetic program [1]. Any impairment in this genetic mechanism(s) during the various phases of the process can lead to infertility. Oxidative stress has been known to be the focus of interest as a potential cause of male infertility [2]. Production of oxygen radicals causes peroxidative damage to spermatozoa, leading to DNA damage, loss of motility and reduction in the fusion capacity of spermatozoa [3].

Selenium (Se) has been found and accepted as an essential micronutrient and a potent chemopreventive agent. It exerts its biological activity in the form of selenoproteins, which have a plethora of functions. Selenoproteins, such as glutathione peroxidase (GSH-Px), thioredoxin reductase, selenoprotein P and selenoprotein W, have been reported to play a role in protecting cells against free radicals and oxidative stress [4]. In testes, Se, an essential trace element, is of fundamental importance, as any deficiency in its nutritional levels reduces testicular mass and morphology, besides causing other flagellar defects in sperms [5]. Even the supranutritional levels of Se can cause severe abnormalities in sperms [6] due to increased oxidative stress. Deficiency in Se causes oxidative stress in testes due to the diminished antioxidant property of this element as part of GSH-Px [7]. Se deficiency has been associated with an increased expression of genes involved in oxidative stress [8]. Reports suggest that at physiologically altered concentrations, Se causes cell cycle arrest and increased apoptotic activity by generating reactive oxygen species (ROS) [9]. A number of epidemiological studies on the effects of low and

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high Se on physiological and pathological conditions in different life forms have been addressed in the past few decades. These studies suggest adverse reproductive and developmental effects in domestic animals, damage to the liver and kidneys, and impaired immune responses following variations in dietary Se levels [10–12]. Epidemiological studies in humans have shown a relationship between chronic oral exposure to Se and an increased incidence of death due to neoplasms [13,14].

Apoptosis and cell cycle regulation are closely associated in proliferating cells [15]. Since cell cycle is coordinated by several protein kinases composed of cyclin-dependent kinase (CDK) subunits and their corresponding regulatory cyclin subunits, they are more likely to influence the process of cell death.

CDC2 (also known as CDK1) and cyclin B1 represent the most important set of proteins regulating cell cycle progression [16]. In all systems, progression to metaphase is controlled by the activity of M-phase promotion factor, which is a heterodimer complex of CDC2 and cyclin B1 [17]. In the case of male gamete formation, these proteins are of utmost importance due to their essential role in the completion of meiosis [18]. Any disruption or inactivation of these proteins can lead to noncompletion of the process and to apoptosis in germ cells [19,20].

Cyclin B1 has been known as a key regulator of apoptosis in response to DNA damage [21]. However, the exact biochemical mechanism by which germ cell apoptosis occurs has not been elucidated [22]. Even the contribution of CDC2 expression or activity to DNA-damage-induced apoptosis is also uncertain [19]. The potential role of CDC2/cyclin B1 in apoptosis has been found to depend upon the system under study [23]. The literature related to the apoptotic ability of Se compounds at varying concentrations and forms (both organic and inorganic) is essentially inconclusive [24].

To obtain further insights into the function of Se at nutritional concentrations in cell cycle progression, the present work aimed to study the influence of oxidative stress caused by variations in dietary Se on the expression pattern of CDC2 and cyclin B1 of mouse testes in terms of mRNA and protein expressions. The study was further extended to correlate the effect of these alterations in cell cycle regulators with the apoptotic activity of germ cells during spermatogenesis in terms of the expression of Bcl-2, Bax, caspase-3 and caspase-9, which represents the intrinsic and extrinsic pathways of apoptosis.

2. Materials and methods

2.1. Chemicals

Sodium selenite (Na_2SeO_3), 2,3-diaminonapthalene (DAN) and dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRI reagent and one-step reverse transcriptase–polymerase chain reaction (RT-PCR) kit were obtained from Molecular Research Center, Inc. (Cincinnati, OH) and Qiagen, respectively. Oligonucleotides were synthesized by Sigma-Aldrich. PVDF membrane was purchased from Millipore (USA), whereas antibodies to CDC2 and cyclin B1 were from Rockland (Pennsylvania) and Sigma-Aldrich, respectively. For CDC2 kinase assay, histone H1 was obtained from Sigma-Aldrich, and [γ-^{32}P]ATP (specific activity=4800 Ci/mmol) was obtained from Bhabha Atomic Research Center (Mumbai, India).

2.2. Animals and experimental design

Six-week-old male Balb/c mice with a body weight of approximately 25 g were procured from the Central Animal House, Panjab University (Chandigarh, India). All experiments and protocols were approved by the institutional animal ethical committee. Animals were acclimatized to departmental animal rooms for a period of 1 week prior to the start of 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 a special yeast-based diet for a period of 8 weeks.

2.3. Diet preparation

A yeast-based synthetic Se-deficient diet (approximately 0.02 ppm Se) was prepared in the laboratory according to the composition given by Burk [25]. It contained torula yeast (inactivated), 30%; sucrose, 56.99%; corn oil, 6.67%; mineral mix, 5%; vitamin mix, 1%; DL-methionine, 0.3%; vitamin E, 0.04%. Se-adequate and Se-excess diets were prepared from Se-deficient diet by supplementing it with 0.2 and 1 ppm Se, respectively, as Na_2SeO_3. After completion of the diet feeding schedule of 8 weeks, animals were sacrificed under barbitone intoxication, and testes were removed and used for various analyses.

2.4. Se levels

Se levels were estimated in testes using the method of Hasunuma et al. [26]. The assay is based on the principle that Se content in samples is converted to selenous acid on acid digestion. The reaction between selenous acid and aromatic-*o*-diamines, such as DAN, leads to the formation of 4,5-benzopiazselenol, which displays brilliant lime-green fluorescence when excited at 366 nm in cyclohexane. Fluorescence emission in extracted cyclohexane was read on a fluorescence spectrophotometer using 366 nm as excitation wavelength and 520 nm as emission wavelength.

2.5. Biochemical estimations

Tissue homogenates were prepared (10% wt/vol) in 50 mM Tris–HCl (pH 7.4) using mechanically driven Teflon-fitted potter elvejham-type homogenizer under ice-cold conditions. The homogenates were then subjected to centrifugation at 10,000 rpm for 30 min. The supernatant

Table 1
Primer pairs used

Gene	Primer	Reference
CDC2	Sense 5′-GAA AGC GAG GAA GAA GGA GTG-3′ Antisense 5′-CAA GCA GTT CTC GTC CAG GTT-3′	Accession no. AF488732
Cyclin B1	Sense 5′-ATC GGG GAA CCT CTG ATT TT-3′ Antisense 5′-TCA CAC ACA GGC ACC TTC TC-3′	[31]
Bcl-2	Sense 5′-AGA GGG GCT ACG AGT GGG AT-3′ Antisense 5′-CTC AGT CAT CCA CAG GGC GA-3′	[32]
Bax	Sense 5′-GGT TTC ATC CAG GAT CGA GAC GG-3′ Antisense 5′-ACA AAG ATG GTC ACG GTC TGC C-3′	[33]
Caspase-3	Sense 5′-AGT CAG TGG ACT CTG GGA TC-3′ Antisense 5′-GTA CAG TTC TTT CGT GAG CA-3′	[34]
Caspase-9	Sense 5′-TGC ACT TCC TCT CAA GGC AGG ACC-3′ Antisense 5′-TCC AAG GTC TCC ATG TAC CAG GAG C-3′	[35]

[postmitochondrial fraction (PMF)] thus obtained was collected, and various biochemical estimations were performed as described below.

2.6. Se-dependent GSH-Px activity

GSH-Px activity was assayed by the coupled enzyme procedure with glutathione reductase using H_2O_2 as substrate [27]. The assay was carried out in the PMF of testes. The activity was expressed as micromoles of NADPH oxidized per minute per milligram of protein. The total protein estimation was performed using the method of Lowry et al. [28].

2.7. Lipid peroxidation (LPO) assay

The levels of LPO were assayed using the method of Wills [29]. Since malondialdehyde (MDA) is a degradation product of peroxidized lipids, the development of pink color with the absorption characteristics (absorption maxima at 532 nm) of TBA-MDA chromophore is taken as an index of LPO. As standard, 2–10 nmol of 1,1′,3,3′-tetraethoxypropane was used; for controls, distilled water was used instead of the sample. MDA levels were expressed as nanomoles of MDA per milligram of protein.

2.8. Determination of free radicals in testes

Determination of free radicals was based on the modified method of Driver et al. [30]. This method is based on the principle that the acetate group of DCFH-DA will be cleaved by esterases activated by ROS to form a fluorescent product dichlorofluorescein (DCF), whose intensity can be measured at 530 nm. Testicular homogenates were prepared in ice-cold Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 10 mM D-glucose and 5 mM HEPES, pH 7.4). The homogenates were allowed to warm at 21°C for 5 min. The reaction mixture containing 10 μM DCFH-DA and 5 mg/ml tissue was incubated for 15 min at room temperature (21°C) to allow the probe to be incorporated into any membrane-bound vesicle and to allow diacetate groups to be cleaved by esterases. After another 30 min of incubation, the conversion of DCFH to the fluorescent product DCF was measured with a fluorescence spectrophotometer, with excitation wavelength at 485 nm and emission wavelength at 530 nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks. The relative difference in fluorescence intensity was taken as the measure of the amount of ROS in different treatment groups.

2.9. RNA isolation and mRNA expression of CDC2 and cyclin B1 using RT-PCR

Total RNA from testis was extracted with TRI reagent (Molecular Research Center, Inc.). The integrity and the size distribution (quality) of RNA were examined on 1.2% formaldehyde agarose gel electrophoresis.

The mRNA expression of CDC2 and cyclin B1 was analyzed in testes using the RT-PCR kit from Qiagen. Two micrograms of RNA from each group was used in the

Table 2
Effect of different Se statuses on GSH-Px activity and LPO after the 8-week diet feeding schedule

	Group I (Se-deficient)	Group II (Se-adequate)	Group III (Se-excess)
Se estimation (μg Se/g tissue)	0.666±0.073**	0.938±0.023	1.203±0.092**
LPO (nmol MDA/mg protein)	0.724±0.063**	0.302±0.035	0.608±0.042**
GSH-Px (μmol NADPH oxidized/min/mg protein)	84.29±3.76**	125.871±5.01	148.09±9.36*
Free radical production (relative fluorescent intensity of DCF)	95.40±8.67**	27.42±2.42	73.01±4.03**

The values are presented as the mean±S.D. of six independent observations.
* $P<.01$.
** $P<.001$.

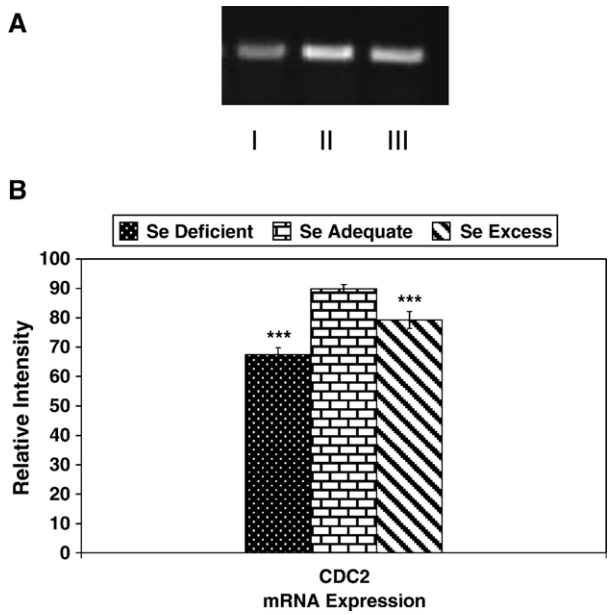

Fig. 1. Effect of Se-induced oxidative stress on the mRNA expression of CDC2 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *$P<.05$, **$P<.01$ and ***$P<.001$.

RT-PCR mixture prepared as per the manufacturer's guidelines. The contents were mixed gently, and PCR was performed in a thermal cycler (Techne Ltd., England) using the following conditions: RT reaction at 50°C for 50 min, initial PCR activation performed at 95°C for 15 min, followed by 35 cycles of 94°C (denaturation) for 45 s, 58°C (annealing) for 45 s and 72°C (extension) for 1 min, and final incubation at 72°C for 10 min to extend any incomplete strand. To bring RT-PCR nearer to the quantitation parameter, initially, the reaction for RNA from Group II was carried at 20, 25, 30 and 35 cycles; progressively increasing product concentrations were observed, hence the 35-cycle RT-PCR was used in further experiments.

The list of primer sequences used for the analyses of various genes is shown in Table 1. The final PCR products formed were analyzed on 1.5% agarose gel electrophoresis, and densitometric analysis of bands was performed with Image J software (NIH, USA).

2.10. Western immunoblot analysis

Protein samples (40 μg) from each treatment group were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrophoretically transferred to PVDF membranes (Millipore). Immunoblot was prepared using anti-mouse anti-CDC2 primary antibodies (Rockland), anti-cyclin B1 primary antibodies (Sigma-Aldrich) and peroxidase-conjugated anti-rabbit IgG secondary antibodies (Sigma-Aldrich). A diaminobenzidine (DAB)+H_2O_2 detection system was used to develop blots.

2.11. Immunohistochemical analysis for CDC2 kinase and cyclin B1

Deparaffinized sections were incubated with 3% H_2O_2 at 37°C to block endogenous peroxidase activity. Sections were then blocked with 2% bovine serum albumin (BSA) for 30 min at 37°C and incubated with anti-CDC2 kinase and anti-cyclin B1 primary antibodies, respectively (1:200), diluted in PBS containing 1% BSA. Slides were dipped in 0.05% Tween-20 in PBS and incubated with peroxidase-conjugated secondary IgG for 45 min. The reaction product was developed using DAB+H_2O_2. Sections were then mounted on glycerol jelly.

2.12. Preparation of protein extracts for kinase assay

Testes were collected from the different treatment groups after the completion of the 8-week diet feeding schedule. Total lysates were prepared in fresh ice-cold protein lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40, 50 mM NaF, 1 mM Na_3VO_3 and 1 mM PMSF). Extracts were cleared by centrifugation at 440×g for 10 min at 4°C. The supernatants were collected for in vitro CDC2 kinase assay. The protein concentrations of testis extracts were determined using the method of Lowry et al. [28].

2.13. Immunoprecipitation and CDC2 kinase assay

Histone kinase assay was performed as described by Zhu et al. [18]. For immunoprecipitation, 10 μg of total proteins from precleared lysate was incubated with specific antibodies to CDC2 overnight at 4°C with shaking. Protein A-Sepharose was added and incubated

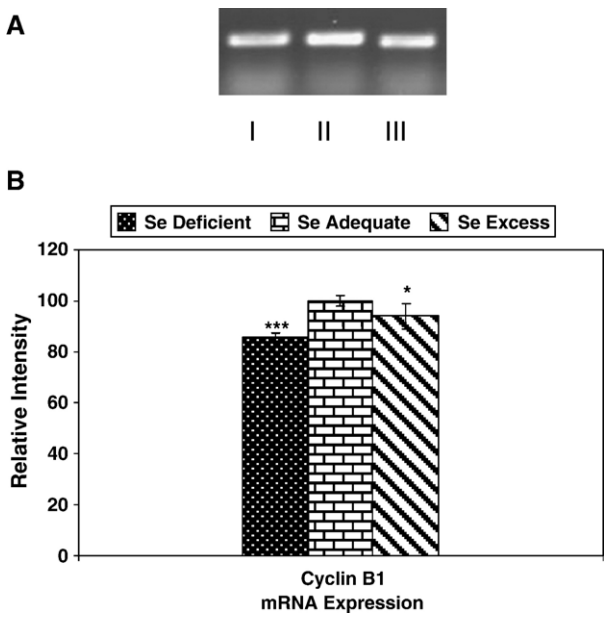

Fig. 2. Effect of Se-induced oxidative stress on the mRNA expression of cyclin B1 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean ±S.D. of four independent observations. *$P<.05$, **$P<.01$ and ***$P<.001$.

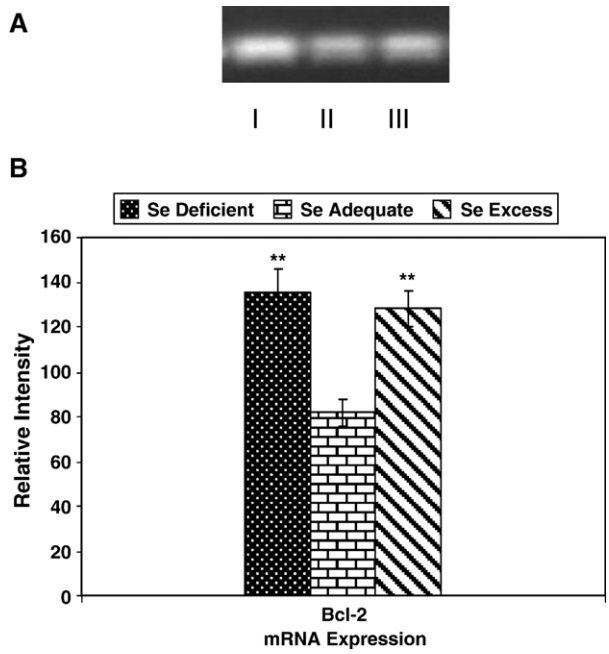

Fig. 3. Effect of Se-induced oxidative stress on the mRNA expression of Bcl-2 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *P<.05, **P<.01 and ***P<.001.

for 2 h at 4°C with shaking. Immunoprecipitates were collected by centrifugation, and pellets were washed four times with 750 μl of ice-cold immunoprecipitation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA pH 8.0, 1 mM PMSF).

Immunoprecipitated complexes were collected and washed thrice with ice-cold kinase assay buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 1 mM DTT and 10 mM ATP). Histone H1 kinase activity was assayed in kinase assay buffer using 10 μg of calf thymus histone H1 and 0.1 mCi/ml [γ-^{32}P]ATP. Reactions were incubated at 30°C for 30 min and terminated by the addition of an equal volume of 2× SDS-PAGE sample buffer. Samples were heated for 1 min in boiling water and then separated on 12% SDS-PAGE, stained with Coomassie blue, dried, exposed to X-ray film (X-OMAT-AR) and developed.

2.14. Terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) assay for the detection of apoptotic germ cells

TUNEL was carried for in situ visualization of DNA fragmentation indicative of apoptosis using an in situ cell death detection kit (Roche Diagnostics, Germany) on 7-μm-thick paraffin-embedded testis sections on glass slides. After proteinase K treatment, slides were treated with TUNEL reaction mixture containing label solution and enzyme solution. Thus, 3′-OH DNA ends were labeled with biotin-conjugated dUTP and detected with avidin conjugate. To negative controls, label solution without any enzyme solution was added. For positive controls, 50 μl of DNase I (1500 U/ml) was added for 10 min at 25°C, and then TUNEL reaction mixture was added.

2.15. Statistical analysis

Data are expressed as the mean±SD of at least six independent observations for all biochemical estimations. Differences between different groups were 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).

3. Results

3.1. Se levels

Se levels estimated in the testes of different Se-supplemented groups after 8 weeks of the diet feeding schedule are shown in Table 2. A significant decrease (29%; P<.001) in Se concentration in the testes of animals supplemented with Se-deficient diet (Group I) was obtained, compared with that in Group II animals. Similarly, in the Group III animals supplemented with Se-excess diet (1 ppm), a significant increase (28%; P<.001) in the levels of this trace element was recorded, as compared with that in the respective adequate group (Group II).

3.2. GSH-Px activity

There was a significant decrease in the activity of GSH-Px in the testes of the Se-deficient group (Group I; P<.001) as compared with that in the Se-adequate group (Table 2).

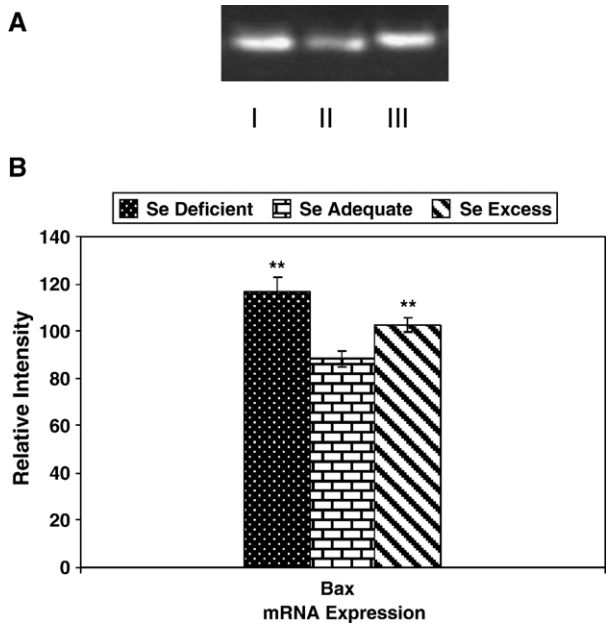

Fig. 4. Effect of Se-induced oxidative stress on the mRNA expression of Bax in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *P<.05, **P<.01 and ***P<.001.

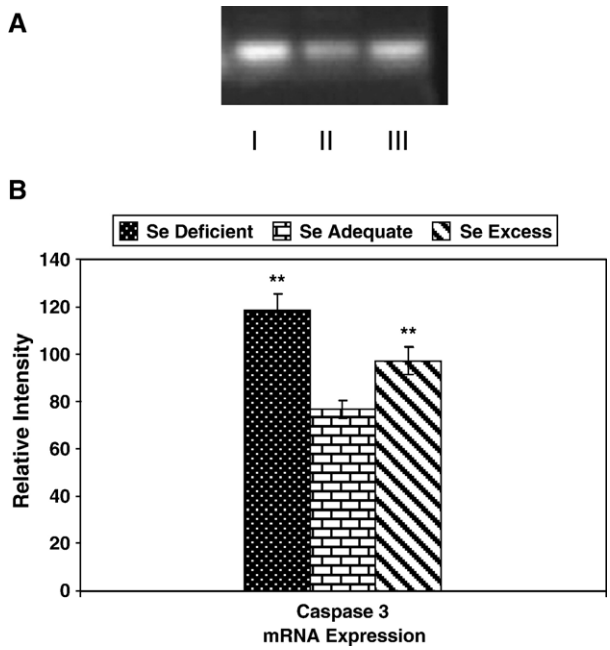

Fig. 5. Effect of Se-induced oxidative stress on the mRNA expression of caspase-3 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *P<.05, **P<.01 and ***P<.001.

However, a significant increase in enzyme activity was seen in the Se-excess group (P<.01) as compared to the corresponding Se-adequate group.

3.3. LPO

The MDA levels as presently observed indicate the LPO level in tissues (Table 2). A highly significant increase (P<.001) in LPO was recorded in the testes of both Se-deficient and Se-excess animals compared with that in animals fed an Se-adequate diet.

3.4. Evaluation of free radicals in testis

There was a significant increase in radical production in Group I animals as compared to Group II animals, indicating the induction of oxidative stress in this group (Table 2). Similarly, in Group III animals fed on Se-excess diet, an increase in ROS levels was observed. However, this increase was less than that in Group I (Se-deficient) animals.

3.5. mRNA expression of CDC2, cyclin B1 and genes involved in apoptosis

Alterations in the mRNA expression of CDC2, cyclin B1, Bcl-2, Bax, caspase-3 and caspase-9 following Se-induced oxidative stress are shown in panels A and B of Figs. 1–6. A significant decrease in the mRNA expression of both CDC2 and cyclin B1 was observed under conditions of Se deficiency and Se excess, as compared to the respective Se-adequate group. However, an increase in the mRNA expression of a few genes involved in apoptosis, such as *Bcl-2*, *Bax*, *caspase-3* and *caspase-9*, was observed in Groups I and III. These observed changes in expression patterns were more prominent in the Se-deficient group. It was also observed that CDC2 is more susceptible to alterations by Se-induced oxidative stress than its regulatory counterpart, cyclin B1.

3.6. Western blot analysis for CDC2/cyclin B1

Western blot analysis was performed to study changes in the protein expression of CDC2 and cyclin B1 in response to oxidative stress (Fig. 7A and B; Fig. 8A and B). Alterations in protein expression were found to show similar trends as were observed in the mRNA expression of both cell cycle regulators (i.e., CDC2 and cyclin B1). A decrease in the expression of CDC2 and cyclin B1 was seen in both Se-deficient and Se-excess groups. As already shown in the case of mRNA expression, this decrease is outstanding in the Se-deficient group, and especially in CDC2.

3.7. Immunohistochemistry of CDC2 kinase and cyclin B1

The immunohistochemical localization of CDC2 kinase and cyclin B1 in testis sections is shown in Figs. 10 and 11, respectively. The expression of CDC2 was found to be intense in spermatogonia and spermatocytes. In Group I and Group III animals, the localization of the protein is found to be similar to that in Group II. However, an overall decrease in expression was found as detected by fewer immuno-positive cells in the Se-deficient and Se-excess groups. This decrease in the immunohistochemical localization of

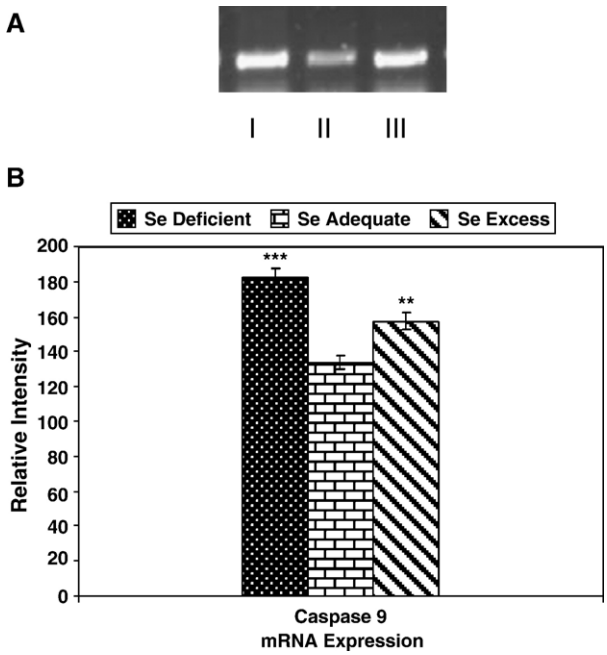

Fig. 6. Effect of Se-induced oxidative stress on the mRNA expression of caspase-9 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *P<.05, **P<.01 and ***P<.001.

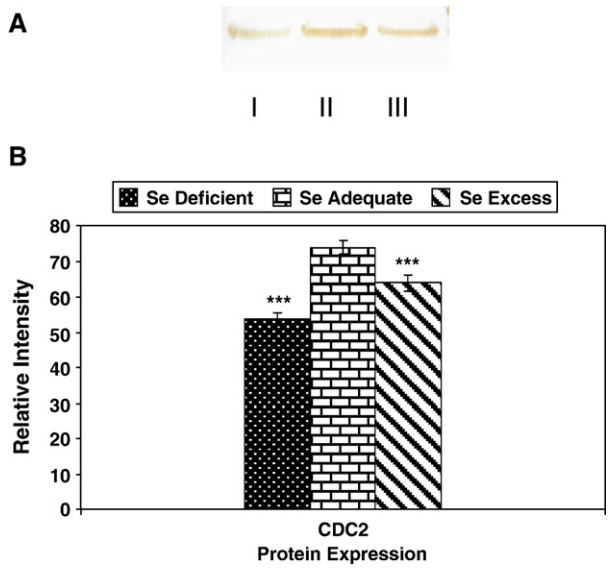

Fig. 7. Effect of Se-induced oxidative stress on the protein expression of CDC2 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *P<.05, **P<.01 and ***P<.001.

proteins was more prominent in Group I (i.e., Se-deficient animals as compared to Se-excess animals).

Cyclin B1 expression in the Se-adequate group (i.e., Group II) was found to be intense in spermatogonia, spermatids and sperm heads, whereas in Groups I and III, the overall expression of the protein was found to be diminished. However, almost no expression was observed in sperm heads. A mild expression was also seen in spermatogonia and spermatocytes.

3.8. CDC2 kinase assay

CDC2 kinase activity was assayed in specific immunoprecipitates from the testes of different treatment groups by its ability to phosphorylate exogenous histone H1. A significant kinase activity was present in CDC2 immunoprecipitates from extracts of the testes of mice in the Se-adequate group (Fig. 9A). However, a significant decrease in kinase activity was detected in immunoprecipitates from the extracts of the testes of Group I animals, as measured by densitometric analysis of bands obtained on autoradiograph (Fig. 9B). Even the Se-excess group also demonstrated a decrease in kinase activity.

3.9. TUNEL assay

TUNEL assay was used to identify and characterize apoptotic germ cells following induction of oxidative stress. The frequency of apoptotic germ cells was greater in the testicular sections of Se-deficient and Se-excess animals (Fig. 12C and E) as compared to the Se-adequate group (Fig. 12D). Spermatogonial cell populations are found to be maximally apoptotic (Fig. 12). However, in Se-deficient animals, there was also a simultaneous increase in TUNEL-positive spermatocytes (Fig. 12C) compared with that in the Se-adequate group where no such DNA damage was observed (Fig. 12D). Apoptosis in the Se-excess group was also noticed through condensation of nuclei (Fig. 12E). Negative and positive controls were run simultaneously to detect and compare apoptotic activities between different groups (Fig. 12A and B).

4. Discussion

Se, a potent antioxidant, has been associated with male fertility [36]. Any alteration in its physiological concentrations leads to abnormal spermatogenesis [5] and, hence, male infertility. In the present study, administration of yeast-based Se-deficient diet led to a significant decrease in testicular Se levels, as has also been reported earlier [37]. The animals supplemented with supranutritional levels of the element above the adequate limit exhibited an increase in the Se concentration. Although the concentration of Se used is more than the normal permissible levels, it is well below the toxic limits [38]. These altered Se concentrations affect oxidative status because of its known antioxidant nature in the tissues.

Se exerts its physiological activity in the form of selenoproteins, such as GSH-Px, thioredoxin reductase, selenoprotein P and selenoprotein W. In the case of testes, the enzyme GSH-Px is the major antioxidant selenoprotein [39]. The PH-GPx isoform of the enzyme GSH-Px contributes maximally to the testes [40]. However, the fact that PH-GPx is nonresponsive to dietary Se concentration variations prompted us to measure the total GSH-Px, which is a good indicator of both Se level and the redox state of tissues [41].

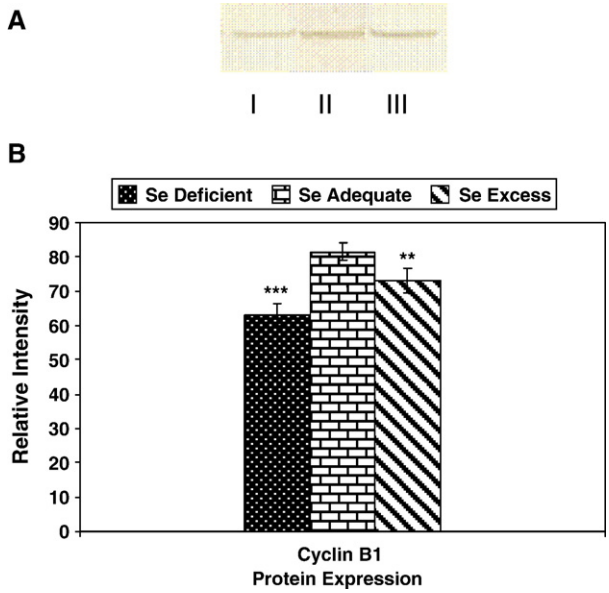

Fig. 8. Effect of Se-induced oxidative stress on the protein expression of cyclin B1 in testes (A) and its densitometric analysis (B). Lane I, Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *P<.05, **P<.01 and ***P<.001.

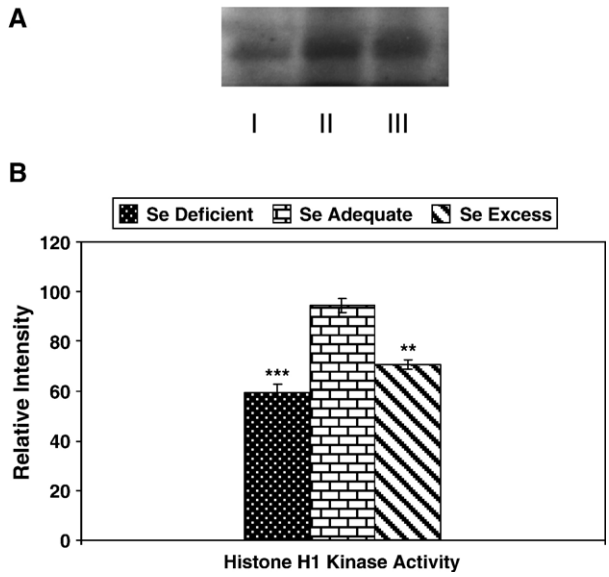

Fig. 9. Effect of Se-induced oxidative stress on in vitro histone H1 kinase activity in testes (A) and its densitometric analysis (B). Lane I; Se-deficient. Lane II, Se-adequate. Lane III, Se-excess. The values are presented as the mean±S.D. of four independent observations. *$P<.05$, **$P<.01$ and ***$P<.001$.

A decrease in GSH-Px activity, as observed in Se-deficient (group I) animals, confirms lowered Se levels and induction of oxidative stress in the testes [5,7]. Recently, a number of workers have reported Se-deficiency-dependent down-regulation of GSH-Px activity [42]. In the Se-excess group (Group III), an increase in GSH-Px activity was observed, as compared to the Se-adequate group (Group II). This change in GSH-Px activity was not as significant as in the case of the deficient group. This may be attributed to the presence of a homeostatic process controlling the levels of GSH-Px so that any higher intake of this element above nutritionally adequate limits does not elicit any further increase in enzyme activity [41]. Se-excess-induced toxicity can be explained on the basis of a free radical mechanism [9,43]. Se at higher concentrations reacts with glutathione to form a highly reactive redox species $GSSe^{-}$, which is a seleopersulfide [44] that reacts with GSH to generate superoxides. It also reacts with GSH catalytically to reduce cytochrome *C* and to induce mitochondrial swelling, a precursor of apoptosis [38].

GSH-Px mainly eliminates peroxides (such as fatty acid peroxides) from cells, thus protecting cells against damage caused by free radicals and products of LPO [45]. Reduced GSH-Px levels in Se-deficient animals show diminished antioxidant defense mechanism and increased oxidative stress.

LPO is one of the most potent indicators of oxidative stress in the case of testes because of the high concentration of polyunsaturated fatty acids and the ability to generate ROS that renders germ cells more susceptible to peroxidative damage [46]. Increased LPO levels and decreased GSH-Px activity in the case of Se-deficient animals confirm the induction of oxidative stress in this group. The Se-excess group demonstrated an increase in levels of MDA indicating increased LPO and, hence, more oxidative stress when compared to the Se-adequate group of animals.

Presently, a statistically significant increase in the levels of free radicals in the case of Se-deficient and Se-excess groups was observed, as compared to the animals supplemented with the Se-adequate diet. A significant increase in ROS and peroxidation inside the cells in the case of Se deficiency has also been reported earlier [47].

There exist several contradictory reports regarding the influence of different Se concentrations on the apoptotic, hence anticarcinogenic, ability of this micronutrient. Although the critical importance of Se in the growth of various cell lines has been reported [48,49], the delineation of Se function at low concentrations in cell cycle is lacking. At supranutritional levels, Se has anticancer effects that are mediated through changes in the proliferation of certain cells, apoptosis and/or toxic effects [50,51]. In contrast, the effects of Se at low concentrations are not obvious because of in vitro studies on different cell lines [52]. In light of this background, the effect of variations in dietary Se levels on two important cell cycle regulators CDC2 and cyclin B1, which regulate the G_2/M-phase transition of cell cycle, was studied. The decrease in the mRNA and protein expressions of both these proteins following a reduced dietary intake of Se is in accordance with the results linking Se deficiency with the down-regulation of CDC2 and cyclin B1 and, hence, cell cycle arrest [52]. Se-excess animals also exhibited a decrease in the expression of these proteins, which can be attributed to the apoptotic and anticarcinogenic effects of Se at higher

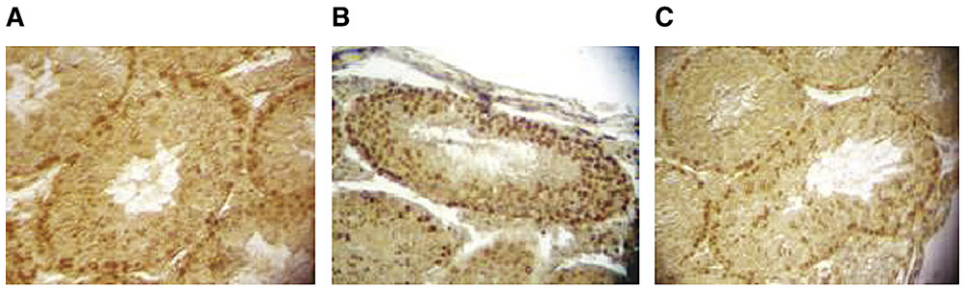

Fig. 10. Immunohistochemistry demonstrating the localization of CDC2 in the testes of different groups fed varying Se concentrations. Se-deficient (A). Se-adequate (B). Se-excess (C).

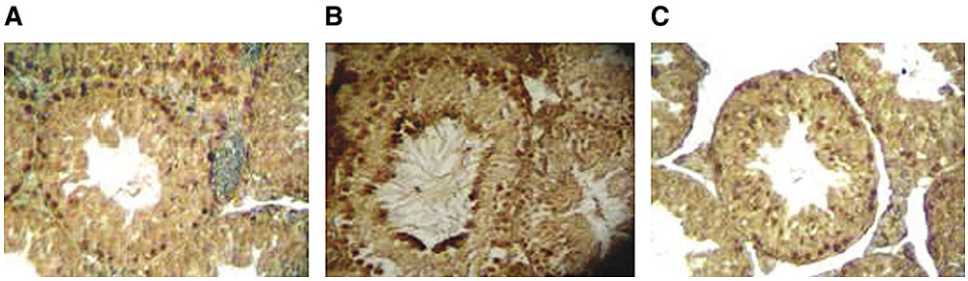

Fig. 11. Immunohistochemistry demonstrating the localization of cyclin B1 in the testes of different groups fed varying Se concentrations. Se-deficient (A). Se-adequate (B). Se-excess (C).

concentrations [50,51]. These findings are further supported by the fact that a decrease in cyclin B1 occurs following DNA damage [53]. The decrease in cyclin B1 levels, as seen in our study, is crucial to determining the levels of CDC2 due to its being the regulator of apoptosis and the activity of the CDC2/cyclin B1 complex. The repression of cyclin B1 transcription thus provides a mechanism for G_2/M-phase arrest [54] in germ cells.

Both cyclin B1 and CDC2 are proteins that show temporal and spatial patterns of expression during cell cycle. Any alteration in their localization can thus provide important information regarding their critical function during the process. Immunohistochemistry was performed to visualize any variation brought about by Se-induced oxidative stress in the localization and expression of these proteins. Under normal conditions, cyclin B1 starts expressing in spermatogonia before meiosis and reaches the maximum in spermatids, as already reported [55]. In the present study, cyclin B1 expression is found to be reduced in Groups I and III as compared to Group II; however, this reduced expression could still be seen in these cells only. These results are in good agreement with decreased protein expression as studied by Western immunoblot. The expression of CDC2 was also found to be decreased in Group I and III animals, as compared to Group II. However, the localization of the proteins remains in spermatogonia and spermatocytes. This can be attributed to the fact that Se-induced oxidative stress in these groups has led to a significant decrease in the expression of this protein. Thus, the overall decrease in the expression of these cell cycle regulators could be referred to the inhibitory effect of oxidative stress on the biosynthesis of these proteins, as suggested earlier in the case of high-temperature-induced alterations in these proteins [56].

The decrease in the expression of CDC2 and cyclin B1 can also be explained on the basis of increased levels of MDA, the product of LPO. Ji et al. [57] reported that increased LPO results in decreased cyclin B1 and CDC2 levels, leading to cell cycle arrest. These findings show that products of LPO are also able to engage in cell cycle checkpoint functions and, hence, apoptosis.

Apoptosis is a strictly regulated process involving a complex network of proteins interlinked by multiple pathways. Extrinsic and intrinsic pathways mainly governed by caspases and the Bcl-2 family of proteins, respectively, are

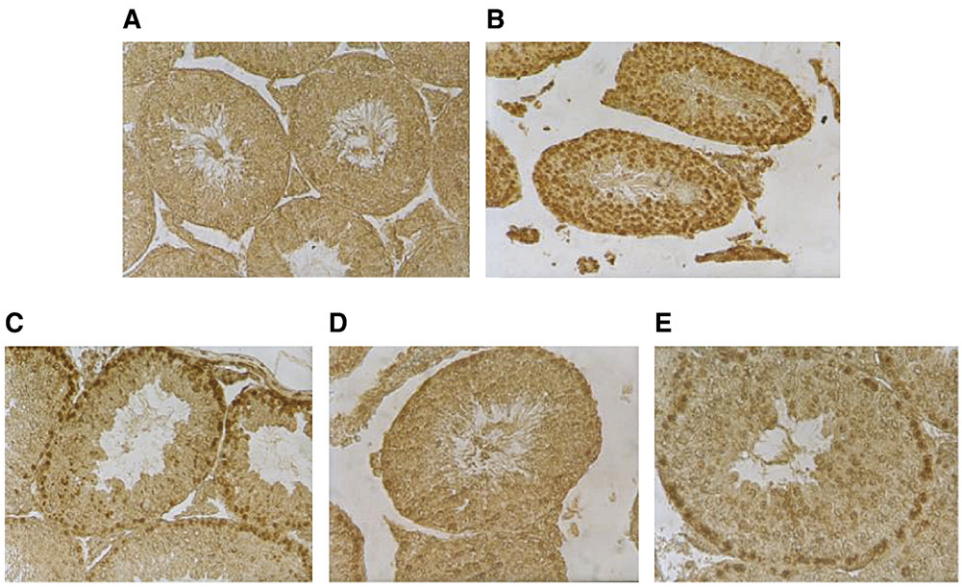

Fig. 12. TUNEL assay in germ cells following alterations in CDC2 and cyclin B1 by Se-induced oxidative stress in different treatment groups. Negative control (A). Positive control (B). Se-deficient (C). Se-adequate (D). Se excess (E).

the most important markers of apoptosis. Alterations in the expression of *Bcl-2* and *Bax* genes indicate the involvement of an intrinsic apoptotic mechanism, whereas caspases point towards the role of an alternative pathway [58]. However, reports regarding caspase-mediated apoptosis are still lacking [59]. In the present study, we found a significant increase in the expression of genes *Bcl-2*, *Bax*, *caspase-3* and *caspase-9*, which are involved in different apoptotic pathways, thus directing towards the molecular mechanism of male germ cell apoptosis in response to alterations in CDC2 and cyclin B1 by Se-induced oxidative stress. There exist reports correlating the levels of seminal ROS with the levels of caspase-3 and caspase-9. It has been found that the expression of caspase-3 and caspase-9 was higher in the semen of infertile men than in the semen of normal healthy donors [60]. Moreover, an increase in the expression of Bax also explains its being a known apoptotic promoter factor [61]. However, an increase in *Bcl-2*, an antiapoptotic gene, points towards the direction of a defense mechanism used to prevent apoptosis in tissues against the oxidative stress induced by Se. Studies also support the fact that Se up-regulates the expression of Bcl-2 and other apoptotic factors [62].

Since the activity of CDC2 defines a threshold level of DNA damage and determines the balance between alternative responses to G_2 arrest and onset of apoptosis, the decrease in CDC2 kinase activity, as seen in Se-deficient and Se-excess animals in response to oxidative-stress-induced DNA damage, reduced the threshold level of damage required to induce apoptosis, as suggested by Ongkeko et al. [19]. Moreover, kinase activity is the actual indicator of the ability of CDC2 to phosphorylate and, thus, activate/inhibit a number of substrates that will be eventually involved in cell cycle regulation. A decrease in CDC2 kinase activity has already been demonstrated in response to Se-induced oxidative stress, leading to the induction of apoptosis [63].

Oxidative stress plays a pivotal role in the induction of apoptotic cell death [64]. However, the exact biochemical mechanism by which ROS-mediated germ cell apoptosis occurs remains to be delineated [22]. Elevated levels of apoptosis are more prevalent during the division of spermatogonia and spermatocytes [65,66]. These findings are concerned with the observed increased apoptosis in the groups supplemented with the Se-deficient and Se-excess diets. Spermatocytes are found to undergo apoptosis in these groups, which can be due to deregulation in spermatogenesis and DNA repair, leading to arrest in this stage of spermatogenesis [67]. This further directs that this stage of spermatogenesis harboring a G_2/M-phase checkpoint actively contributes to the process of spermatogenesis [68].

In conclusion, the present study suggests that Se deficiency and Se excess cause oxidative stress, as can be seen by increased LPO and diminished antioxidant status. This oxidative stress brought about a significant decrease in the expression of CDC2 and cyclin B1, which are the two important cell cycle regulators involved in the G_2/M-phase transition of cell cycle. The decreased expression of these proteins led to cell cycle arrest and, hence, apoptosis in germ cells. Since spermatocytes are found to be TUNEL-positive, it further strengthens the idea that cyclin B1 and CDC2 are affected and that cell cycle arrest occurs, making germ cells unable to differentiate into mature sperms.

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