Biochemical and Biophysical Research Communications 445 (2014) 591–596

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Zinc protects cyclophosphamide-induced testicular damage in rat: Involvement of metallothionein, tesmin and Nrf2

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ARTICLE INFO

Article history:
Received 10 February 2014
Available online 22 February 2014

Keywords:
Zinc
Cyclophosphamide
Metallothionein
Tesmin
Nrf2
Rat testes

ABSTRACT

The role of zinc (Zn) in the protection of germ cells against testicular toxicants has long been elucidated, but the exact molecular mechanisms have not yet been explored. Cyclophosphamide (CP), one of the most commonly used anticancer drugs survived ages of treatment, but the unwanted toxicity limits its clinical usage. The present investigation was aimed to explore the role of Zn and its associated pathways in CP-induced testicular toxicity in S.D. rat. CP was administered in saline 30 mg/kg 5× weekly for 3 weeks (total dose of 450 mg/kg) by *i.p.* route, while Zn was supplemented by oral route at the doses of 1, 3, 10 mg/kg/day for 3 weeks. CP significantly reduced Zn levels in serum and testes, body and testicular weight, sperm count and motility, spermiogenic cells, plasma testosterone and significantly increased the oxidative stress, sperm head abnormalities, sperm DNA damage with decreased chromatin and acrosome integrity; while Zn supplementation ameliorated the same. The present results demonstrated that Zn supplementation protected against CP-induced testicular damages by modulating metallothionein (MT), tesmin and Nrf2 associated pathways. Thus Zn supplementation during anticancer therapy might be potentially beneficial in reducing the off target effects associated with oxidative stress.

1. Introduction

Cyclophosphamide (CP) is a well known alkylating anticancer agent and mainly used in lymphoma, leukemia and for immunosuppresive effects [1]. Its usage is associated with severe adverse effects, in which infertility is one of the major concerns in the younger patients [1,2]. CP-induced reproductive damage was mainly due to the generation of oxidative stress, lipid peroxidation, DNA damage and decreased glutathione levels [3,4]. It has been reported that CP-induced the germ cell damage and several sperm abnormalities along with severe histomorphological changes in the testes of humans and experimental animals [5]. Several intervention studies in experimental models with antioxidants, nutritional supplements were carried out to overcome the reproductive toxicity of CP [6–8], but very few studies delineate the exact molecular mechanisms involved in the testicular damage and the subsequent amelioration by the protective agents. Zinc (Zn) on the other hand is one of the important trace elements in the body, which is a part of more than 300 enzymes required for several vital activities of cell homeostasis, growth and development [9]. Zn deficiency condition in humans is increasing at an alarming rate and leads to multitude of complications [10]. Testes and prostate require high Zn concentrations to maintain their normal physiology. Zn imbalance is proved to cause testicular degeneration and growth retardation [11]. Zn maintains the redox balance by modulating several Zn-dependent enzymes like metallothionein (MT), matrix metalloproteinases (MMPs), Nrf2 and many others [9,12,13]. It has already been proved that the organs where the Zn levels are of prime importance, the deficiency caused disturbance in the redox balance and oxidative stress leading to the cellular damages [14].

Initial experiment on Zn provides the evidence that at appropriate concentration Zn can protect CP-induced damage in the urinary bladder and blood of rat [15]. It has also been suggested that further experiments can explore the underlying mechanisms of Zn protection against CP-induced toxicity. Pharmacological modulation of MT was used as one of the strategies to overcome the toxic issues of several drugs, especially with anticancer agents [16,17]. Previous studies have reported that pharmacological increase in MT levels during anticancer drug regime protects the normal cells from the toxic insults of the drug [16]. It has been reported that tesmin, a Zn bound MT-like protein (MTL5) expressed in the testes in a stage-dependent manner and shuttles between the cytoplasm and nucleus under normal conditions [18].

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http://dx.doi.org/10.1016/j.bbrc.2014.02.055
0006-291X/

Heavy metals like Zn and cadmium (Cd) are thought to influence its expression in the testes of mice [19]. Aberrant change in its expression was linked to oxidative stress [20]. However, tesmin expression under drug induced oxidative stress and damage still remains unexplored. On the basis of the literature it has been observed that CP exposure and Zn deficiency share many common clinical features like induction of oxidative stress, DNA damage, growth retardation, testicular degeneration, delayed maturation, sperm abnormalities, alopecia, anorexia, immunosuppression and delayed wound healing [1,3,21–23]. So it is worthy to explore, whether CP perturbs the Zn homeostasis and thereby increasing the susceptibility of testicular damage and the subsequent influence of external Zn supplementation to ameliorate the same. To investigate the same, rats were injected with CP to induce testicular toxicity and Zn was externally supplemented to decipher (i) the role of Zn in CP-induced testicular toxicity; (ii) whether Zn protects from CP toxicity and alters the expression of Zn-dependent proteins; (iii) to use Zn as a pharmacological modulating agent to induce MT levels. Further, an attempt has been made to address the role of Zn in CP-induced testicular toxicity by taking into account of various other Zn associated parameters.

2. Materials and methods

2.1. Animals

All the animal experimental protocols were approved by institutional animal ethics committee. Male Sprague–Dawley (SD) rats (200–220 g) were procured from central animal facility (CAF), NIPER. The animals were kept at room temperature (22 ± 2 °C), with 50 ± 10% humidity and an automatically controlled 12 h light and dark cycle. Standard laboratory animal feed (purchased from commercial supplier) and water (aquapure) were provided *ad libitum*. Animals were acclimatized for the experimental condition for at least one week before commencement of experiment.

2.2. Dose selection and animal treatment

The study includes six treatment groups, each consisting of 10 rats divided randomly, (1) Control (saline, *i.p.*); (2) Zn control (10 mg/kg/day for 3 weeks, *p.o.*); (3) CP (from Sigma-Aldrich, USA, in saline 30 mg/kg 5× weekly for 3 weeks, *i.p.*, total dose of 450 mg/kg); group 4 (CP + Zn1), 5 (CP + Zn3) and 6 (CP + Zn10) receiving CP (same as group 3) plus Zn as zinc sulphate heptahydrate (from Sigma-Aldrich, USA, 1, 3 and 10 mg/kg/day for 3 weeks respectively, *p.o.*).

2.3. Biochemical parameters

Plasma and serum were properly separated from the terminally collected blood. Testes were dissected free of fat, washed with chilled PBS and immediately processed for biochemical analysis. Malondialdehyde (MDA), reduced glutathione GSH(r), catalase, superoxide dismutase (SOD) were estimated according to protocols described earlier [24]. Plasma testosterone was estimated by ELISA kit (Syntron, Bioresearch, Inc., CA, USA) according to the manufacturer's instruction. Serum and testicular Zn levels were measured at 213.9 nm as described earlier [25] with some modifications using graphite furnace atomic absorption spectrophotometer (GFAAS-5EA, Analytik Jena, Germany). MT levels was estimated using cadmium-hemoglobin saturation assay [26].

2.4. Quantification of histological parameters

Testes were fixed in bouin's solution, dehydrated in ethanol and xylene then embedded in paraffin. 5 μm thick sections stained with hematoxylin and eosin (H&E) and periodic acid-Schiff stain (PAS), for quantification of various types of testicular cells and stages. Further, number of seminiferous tubules/unit area and Johnsen's score were also assessed [27].

2.5. Estimation of cell death and DNA damage

Cell death was assessed by TUNEL assay using commercial kit (Calbiochem, USA). Extent of DNA damage was assessed by halo and comet assays as described previously [28].

2.6. Evaluation of sperm characteristics and protein expressions

Sperm count, sperm motility, sperm head morphology (SHM) were done as described previously [24]. CMA3 staining [29], acridine orange (AO) assay [30], toluidine blue assay [31] were performed to assess the sperm acrosome and chromatin integrity. Nuclear chromatin decondensation (NCD) test was done according to the method described previously [32]. Western blot analysis and IHC (Novolink, leica biosystems, UK) were used to evaluate the protein levels in the testes using primary antibodies against protein of interest and FITC-labeled secondary antibodies (Santa Cruz, USA) [24].

2.7. Statistical analysis

Results are expressed as mean ± SEM for each group. Statistical analysis was performed using Jandel SigmaStat (3.5) statistical software. For multiple comparisons, ANOVA was used and post hoc analysis was performed with Tukey's test. P values $\leqslant 0.05$ was considered significant.

3. Results

3.1. Effect of CP and Zn supplementation on body and organ weight

CP significantly decreased the body weight, which may be attributed to the decreased feed intake. Zn supplementation improved the feed intake and body weight. Testes and epididymis weight were decreased in CP treated groups and Zn supplementation restored the same, but not statistically significant (Supplementary Table 1).

3.2. Effect of CP and Zn supplementation on biochemical parameters

CP significantly increased MDA and decreased GSH(r), catalase and SOD levels including the serum and testicular Zn levels. Zn supplementation significantly brought back all the above parameters to normal in a dose-dependent manner (Fig. 1A–F). Zn has positive correlation with plasma testosterone levels and influences its synthesis [33]. Plasma testosterone level was significantly decreased in CP treatment and increased by Zn supplementation (Fig. 1G).

3.3. Effect of CP and Zn supplementation on testicular histology

Different types of testicular cells were quantified (Supplementary Table 2) and found that CP significantly decreased the number of spermatogonial cells, which is in agreement with the decreased sperm counts (Fig. 1H), but Zn supplementation significantly improved the spermatogonial counts. CP treatment led to distorted and deranged seminiferous tubular structures with dark pyknotic nucleus and giant cells (Fig. 2A). Detachment of spermatogonial cells from the seminiferous epithelium was also observed in CP group. Zn treatment preserved the tubular structure and arrangement of spermatogonial cells in a dose-dependent manner. Further, Zn improved the CP-induced histological alterations as

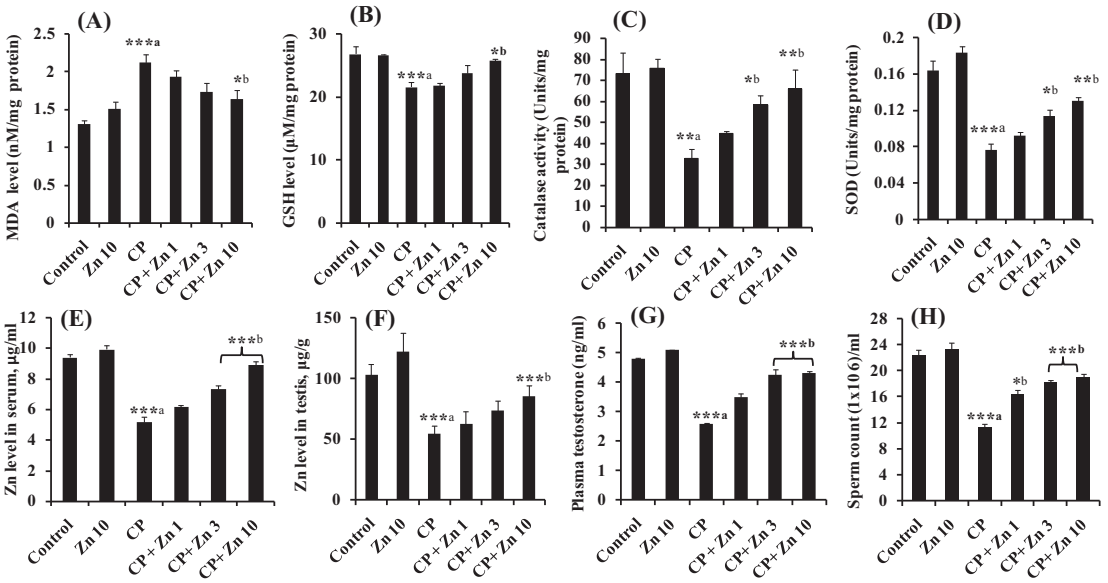

Fig. 1. Effect of Zn and CP on oxidative stress, Zn levels, plasma testosterone and sperm count. CP increased MDA (A); decreased GSH (B), catalase (C), SOD (D), serum and testicular Zn (E and F), plasma testosterone (G) and sperm count (H), while Zn supplementation restored the same. All the values are shown as mean ± SEM, ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 'a' vs. control and 'b' vs. CP.

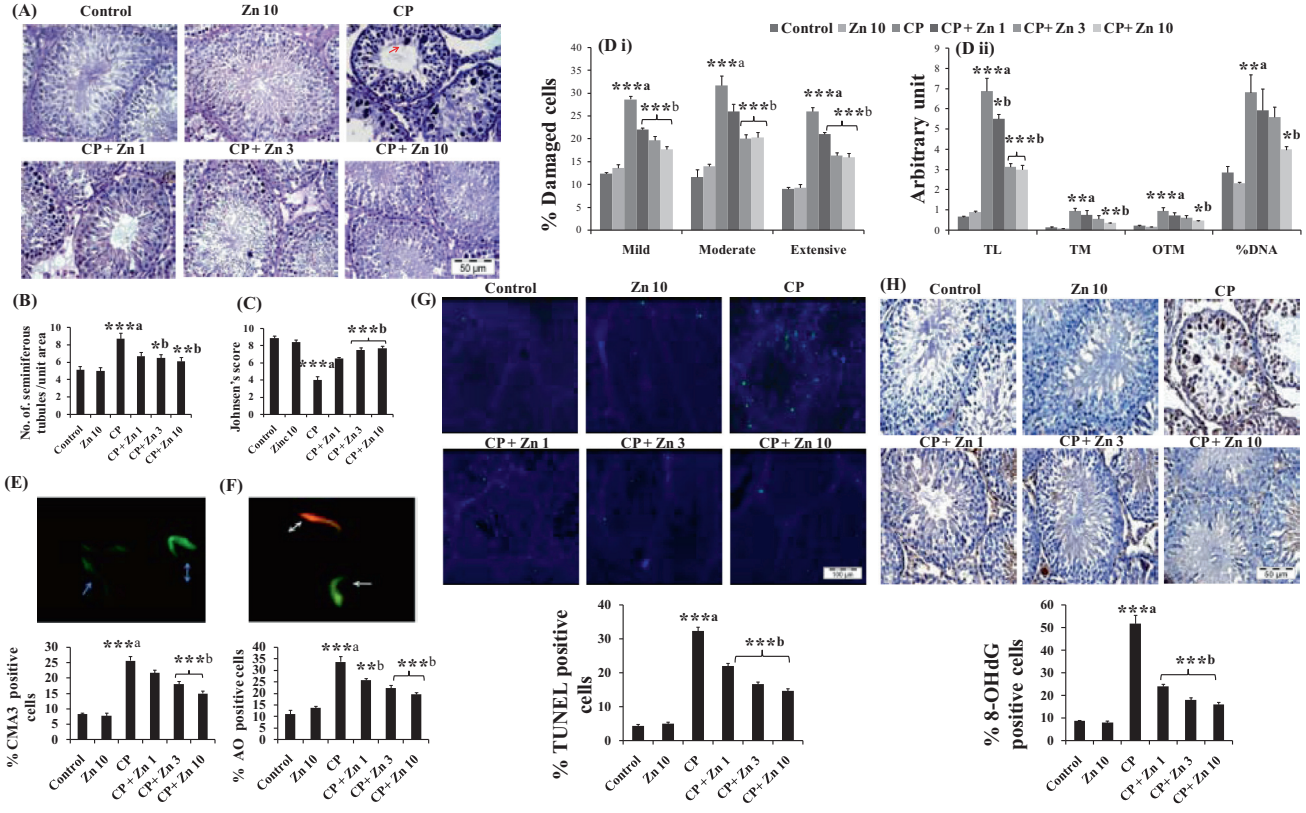

Fig. 2. Effect of Zn supplementation on CP-induced testicular histological changes using PAS staining (A), showed deranged seminiferous tubules with large nucleated bodies and pyknotic cells (indicated by red arrow); while Zn treatment showed protective effects in a dose-dependent manner. Quantification of number of seminiferous tubules/unit area (B), Johnsen's score (C), % DNA damaged cells (Di), comet parameters (Dii), CMA3 and AO positive (E and F) cells (double headed arrows). TUNEL assay (G) and 8-OHdG (H). All the values are shown as mean ± SEM, ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 'a' vs. control and 'b' vs. CP.

observed by number of seminiferous tubule/unit area and Johnsen's score (Fig. 2B and C).

3.4. Effect of Zn supplementation on CP-induced DNA damage in testes and sperm

CP-induced testicular and sperm DNA damage were estimated using halo and comet assay respectively. CP significantly increased number of diffused testicular halos and caused sperm DNA damage. Zn treatment was found to decrease the same in a dose-dependent manner (Fig. 2D).

3.5. Effect of CP and Zn supplementation on sperm characteristics

CP decreased the number of rapid progressive motile sperms (Supplementary Fig. 1A), while Zn maintained the same. Zn reduced the number of abnormal sperm heads induced by CP (Supplementary Fig. 1B). CP treatment increased the number of CMA3 positive (brightly fluoresced) cells, while Zn decreased the same (Fig. 2E). CP, a well known sperm toxicant increased the number of AO positive cells indicative of DNA damage, while Zn protected against the CP-induced damage (Fig. 2F). CP significantly increased deep violet stained sperms with toluidine blue, indicating poor sperm chromatin integrity. Zn supplementation decreased the number of deep violet to light blue sperms; indicating good sperm chromatin integrity (Supplementary Fig. 2A). NCD test was performed in order to assess whether CP effects the sperm decondensation or not. Zn was known to have a positive effect on NCD, by stabilizing the sperm from early decondensation. CP has significantly increased decondensation of sperm than Zn treated groups (Supplementary Fig. 2B).

3.6. Effect of Zn supplementation on 8-OHdG and TUNEL positive cells

TUNEL assay was performed to assess the apoptosis. CP increased the number of testicular apoptotic cells and on contrary Zn reduced the same, providing evidence that Zn can protect against the CP-induced apoptotic damage (Fig. 2G). 8-OHdG was used as a marker, indicative of oxidative DNA damage. The number of 8-OHdG positive cells was significantly increased in CP group, while Zn supplementation reduced the same in a dose-dependent manner in the testes of the rat (Fig. 2H).

3.7. Effect of CP and Zn supplementation on protein expression

MT levels were estimated using Cd-hemoglobin saturation method (Fig. 3Ai) and immunohistochemistry (Fig. 3A). In the present study, MT levels were found to be decreased by CP and external Zn supplementation restored it. Tesmin is a Zn bound protein, expressed specifically in the testes. CP treatment decreased the number of tesmin positive spermatogonial cells (Fig. 3B). Zn supplementation increased the relative expression of tesmin in spermatogonial cells, found both in cytoplasm and nucleus indicating its normal occurrence. CP decreased Nrf2 levels, while Zn increased the same in a dose-dependent manner (Fig. 3C). HO-1 and NQO1 are part of Nrf2 axis and were found to be decreased by CP but Zn ameliorated their expression (Fig. 3D and E). NF-κB a pro-inflammatory mediator known for its notorious nature in the testicular damage was increased by CP treatment, while Zn decreased the same but was not significant (Fig. 3F). 3-β-hydroxysteroid dehydrogenase (3β-HSD) was mainly found in the leydig cells, where prominent steroid synthesis takes place. 3β-HSD was found to be decreased by CP, while Zn treatment improved these levels (Fig. 3G).

4. Discussion

Testes are among the most susceptible organs towards unwanted stress, heat and environmental pollutants [34]. Decreased fertility rate remains one of the challenging tasks to be addressed in younger patients treated with CP [35]. The present experiment reported that CP-induced oxidative stress decreases testicular Zn levels, antioxidant status and various Zn-dependent cellular and biochemical processes. Assessment of Zn in semen was long way considered to be an important factor to assess human reproductive abnormalities [36]. Apart from this serum/plasma Zn assessment is considered to be more suitable and reliable to classify the subjects into various Zn deficient conditions [37,38]. Zhao et al., reported that testicular Zn levels reduced in diabetic mice and also observed that TPEN, a Zn chelator further decreased the Zn levels, which was associated with elevated oxidative stress and apoptosis in testes [25]. CP treatment led to decrease the feed intake which resulted in decrease body weight gain. This was further reflected in decreased testes and epididymis weight, which might be the reasons for delayed testicular growth in rat. Further Zn supplementation improved the feed intake, body and testicular weight. Both serum and testicular Zn levels were found to be significantly decreased by CP treatment. Increased testicular oxidative stress was confirmed by decreased antioxidant enzymes like SOD, GSH and catalase as well as increased MDA level. Zn supplementation ameliorated these changes and improved the testicular antioxidant status. It was reported that at appropriate concentration Zn has positive correlation with sperm motility [39]. In the present study, Zn restored the normal sperm motility and decreased the % of non motile abnormal sperms induced by CP. It was also observed that CP increased the number of abnormal sperm heads and CMA3 positive sperms, which confirmed the altered protamine levels and sperm abnormality. Further, CP-induced various types of sperm DNA damage as observed by comet, AO and toluidine blue assay and Zn supplementation attenuated the same. Histomorphometric analysis and halo assay revealed the testicular cell damage as indicated by increased dark pyknotic cells and reduced number of spermiogenic cells. Further, CP treatment led to decrease sperm counts, plasma testosterone and 3β-HSD levels in the testes. Supplementation with Zn ameliorated the above changes in rat testes, which reconfirmed that Zn has positive relation with them. CP also increased the number of testicular apoptotic cells and 8-OHdG positive cells, which was subsequently decreased by Zn intervention.

The Cd-heme assay and immunohistochemistry confirmed that CP decreased the Zn-dependent enzymes like MT levels in the testes and Zn supplementation restored the same. Increase of MT level by chemomodification was considered to be one of the strategies to protect the off target cells from the toxic insults during chemotherapy [13,40]. Zn and Cd were shown to protect against several alkylating agents by the induction of MT. On the contrary it has also been proposed that Zn showed protection against alkylating agents independent of MT induction and further suggested that a novel Zn-inducible mechanism might play a role [41]. Recently, MTL-5/tesmin a Zn binding protein specifically expressed in testes and play a crucial role in sperm maturation and development [42]. It was reported that change in tesmin expression was shown as an indicator for oxidative stress induced by heavy metals in both *in vitro* and *in vivo* germ cells [19,20]. Tesmin shuttles between cytoplasm and nucleus during normal sperm development, but stress factors could interfere with these dynamics and alter its temporal and spatial expression [18,19]. In the present investigation, it was observed that CP not only altered its dynamics, but also decreased the number of tesmin expressing cells indicative of delayed maturation/testicular growth. So it can be postulated that tesmin might be involved in the protection of

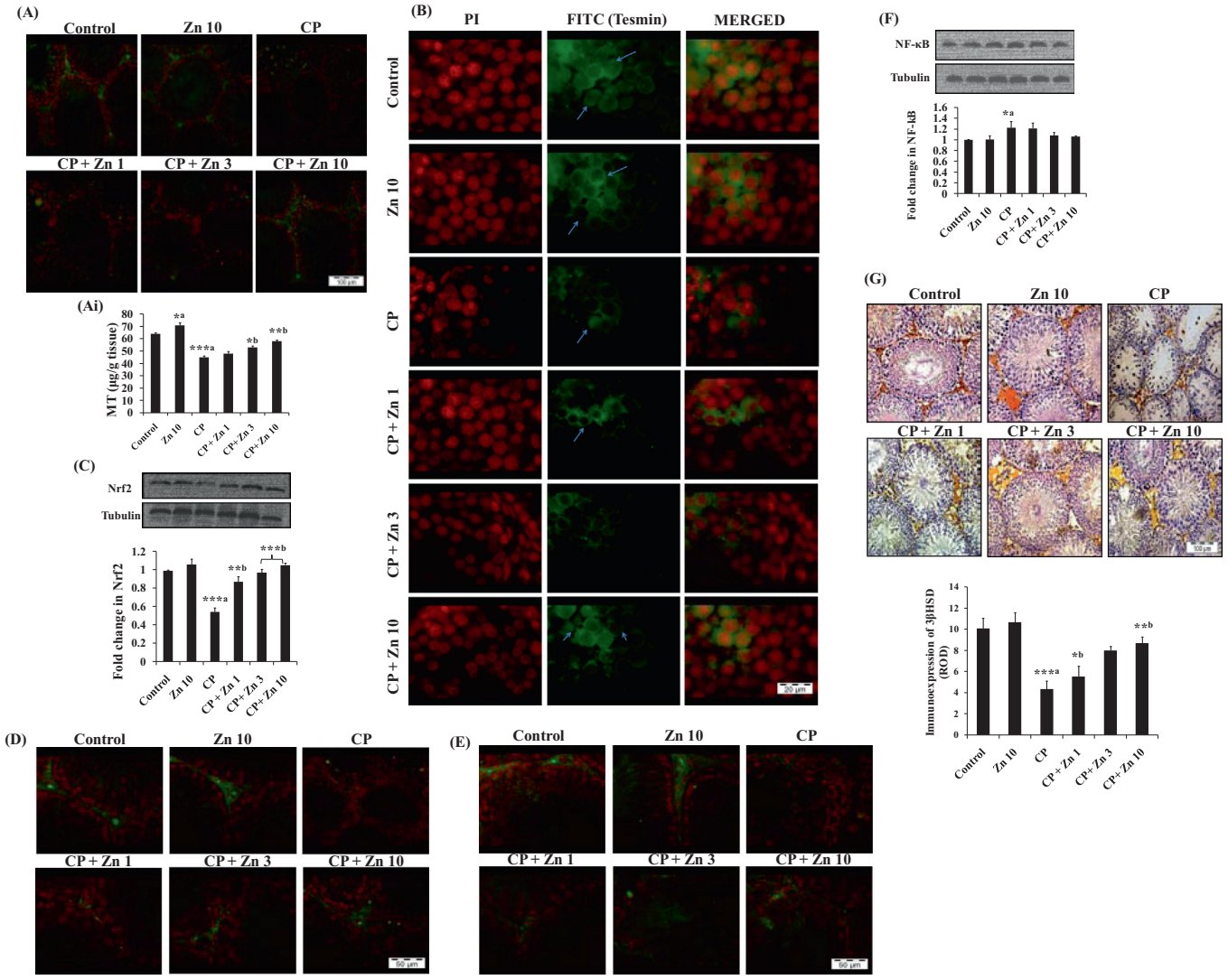

Fig. 3. Effect of Zn supplementation and CP on the expression of MT (A and Ai), tesmin (B) (indicated by arrows), Nrf2 (C), HO-1 (D), NQO1 (E), NF-κB (F) and 3-βHSD (G). Tesmin was found both in cytoplasm and nucleus. HO-1 and 3-βHSD were mainly found in the leydig cells. All the values are shown as mean ± SEM, ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, 'a' vs. control and 'b' vs. CP.

germ cells by Zn supplementation in CP exposed animals. Nrf2 was the main antioxidant system in the body as well as in testes and coordinates various antioxidant processes [43]. Zn also a part of antioxidant system, was reported to act by modulating Nrf2 [12]. Nrf2 was also reported to cause resistance to anticancer drugs in human lung cancer as seen with MT [44]. It has been observed that CP decreased the Nrf2 levels in the testes. Further, Nrf2 downstream pathway molecules such as NQO1 and HO-1 were also perturbed with CP treatment. It is interesting to mention that Zn significantly restored the Nrf2 level including the downstream pathway molecules. This was in consistent with our earlier observation with CP-induced testicular damage and the subsequent intervention with astaxanthin [7]. CP further increased the pro-inflammatory marker NF-κB and Zn decreased the same, but not significantly.

The present study reconfirmed the role of Zn as one of the essential factors in sperm and testicular development. Further studies are needed to decipher the exact involvement of MT, tesmin and Nrf2 pathways in the protection of testicular damage with different classes of anticancer drugs. Thus Zn supplementation during anticancer therapy might be potentially beneficial in reducing the off target effects associated with oxidative stress.

Conflict of interest

None.

Acknowledgments

We wish to acknowledge the financial assistance received from National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, India for carrying out the above experimentation. The authors would like to acknowledge Mr. B. Mallikarjun (Central instrumentation facility, NIPER) for his assistance in the Zn analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.055.

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