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

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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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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