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Ethanolic extract of *Moringa oleifera* leaves alleviate cyclophosphamide-induced testicular toxicity by improving endocrine function and modulating cell specific gene expression in mouse testis

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ABSTRACT

Ethnopharmacological relevance: Moringa oleifera Lam. is known for its nutritional and ethno medicinal values due to the presence of wide array of phytochemicals with multiple biological activities. We have previously reported that ethanolic extract of *Moringa oleifera* leaves (MOE) ameliorated cyclophosphamide (CP)-induced testicular toxicity and improved functional integrity of spermatozoa as well as spermatogenic cells.

Aim of the study: The present study was planned to investigate whether the mitigation of CP-induced testicular toxicity by MOE is mediated via modulation of endocrine profile, genes associated with function of different cell types and enhancement of DNA repair response in spermatogonial cells.

Materials and methods: Adult Swiss albino mice (8 week) were injected with CP (100 mg/kg, one dose in a week for 3 weeks) and MOE (100 mg/kg, 5 doses in a week for 4 weeks) either alone or in combination intraperitoneally. At 35 day post CP injection (first dose), the functional characteristics such as count, motility, head morphology and DNA integrity were assessed in epididymal spermatozoa. Key reproductive hormones like testosterone, follicle stimulating hormone (FSH) and Inhibin B concentration were analyzed in serum and testis. In addition, mRNA expression of genes pertaining to the function of Leydig, Sertoli and spermatogonial cells as well as antioxidant enzymes were evaluated in the testis. To understand the DNA damage and repair process in germ cells, prepubertal (2 week) mice were administered with single dose of CP (200 mg/kg) and/or MOE (100 mg/kg) and analyzed for expression of DNA damage (γ-H2AX, P53 and Caspase3) and repair genes (Rad51 and Ku80) in isolated spermatogonial cells at various time points after treatment.

Results: CP administration resulted in decrease in count, motility and increase in morphological defects and DNA damage in spermatozoa. Testosterone level was marginally decreased while there was a significant increase in FSH ($p < 0.001$) and decrease in inhibin B ($p < 0.05$) observed in CP treated mice. Administration of MOE prior to CP, improved sperm functional characteristics, decreased FSH and increased inhibin B levels. Expression of Abp was down-regulated while Transferrin, Fshr and Gata4 (Sertoli cell specific genes) were up-regulated in testis treated with CP. Administration of CP down-regulated the expression of Oct4 and Ddx4 (Spermatogonia specific genes). MOE administration was shown to ameliorate CP-induced damage by modulating the expression of genes specific to Sertoli and spermatogenic cells. Furthermore, MOE treatment reduced CP-induced DNA damage as evident from lower percentage of γ-H2AX positive spermatogonial cells.

Conclusion: Administration of MOE mitigated CP-induced testicular damage by improving blood and, intratesticular hormonal milieu as well as modulating the expression of genes pertaining to Sertoli and spermatogonial cells.

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1. Introduction

Ideal chemotherapy should target cancer cells without affecting normal cells. Unfortunately, due to the unavailability of such cancer cell-specific treatment, normal tissue toxicity is inevitable thus causing wide spectrum of acute and late health complications. Alkylating agent like cyclophosphamide (CP) is extensively used to treat cancer and immunological disorders. However, due to its non-specific action, CP targets rapidly proliferating tissues like testis which can potentially lead to sub-fertility or infertility in cancer survivors (Kort et al., 2014). Hence, preventing the testicular dysfunction has become a challenging and an important task in cancer treatment.

Spermatogenesis is a highly complex and tightly controlled process along the hypothalamo-pituitary-testicular axis. Anticancer agents are known to perturb spermatogenesis by targeting testicular-endocrine axis (Howell and Shalet, 2002). Studies on rodents and human subjects have revealed that chemotherapeutic drugs alter Follicle stimulating hormone (FSH), Leutinizing hormone (LH) and inhibin B without affecting or causing subnormal testosterone level (Stuart et al., 1990; Wallace et al., 1997). However, degree of toxicity is dependent on nature of chemotherapy regimen, dose and duration of treatment. The administration of bleomycin, etoposide and cisplatin has shown to decrease the expression of steroid acute regulatory protein (StAR) and inhibition of steroidogenic enzymes thereby decreasing testosterone (Al-Bader and Kilarkaje, 2015) in rodents.

The intricate communication between different cell types in testis such as Sertoli, Leydig and spermatogenic cells ensures the normal spermatogenesis. The interactions between the cells are regulated by paracrine and autocrine factors like hormones, growth factors, proteins and enzymes. It has been shown that anticancer drugs can induce testicular dysfunction by perturbing the secretion of these factors, inducing DNA damage and causing impairment in repair process in germ cells (Stukenborg et al., 2018). Therefore, prevention of endocrine dysfunction and mitigation of DNA damage in spermatogenic cells against chemotherapy could be a promising strategy in preserving fertility potential during cancer treatment.

Moringa oleifera Lam. commonly known as drumstick tree, is known to possess immense value in nutrition (Matic et al., 2018) and folk medicine (Senthilkumar et al., 2018) owing to the presence of wide range of nutrients and biologically active phytochemicals (Anwar et al., 2007; Vergara-Jimenez et al., 2017). The plant was reported to utilize in the era of Indian, Greek and Egyptian civilization for improving the skin health and mental fitness. Maurian warriors from India were reported to use Moringa decoction to relieve from the pain, stress and boost the energy during the war (Senthilkumar et al., 2018). Almost all parts of the plant especially leaves, pods and roots have shown tremendous medicinal property which has been exploited in combating a plethora of diseases such as cancer, infection, diabetes, allergy, ulcer, oxidative stress, hypertension, dyslipidemia, neurological problems etc (Al-Asmari et al., 2015; Ekong et al., 2017; Mbikay, 2012; Moura et al., 2015; Uma et al., 2010). A recent study on transcriptomic profile of the plant revealed the candidate genes involved in synthesis the bioactive molecules reflecting medicinal value (Pasha et al., 2019). Leaves, flower and seeds showed the high expression of genes encoding for the enzymes involved in the biosynthesis of vitamins, secondary metabolites like quercetin, kaempferol and benzylamine. Furthermore, iron transport and calcium storage protein expressions were found to be higher in leaves and roots.

Parts of *Moringa oleifera* plant have shown promising tissue protective property against various diseases and external agents in liver, retina, testis, cardiovascular and nervous system (Kou et al., 2018; Kumar Gupta et al., 2013; Nandave et al., 2009; Sadek, 2013; Vergara-Jimenez et al., 2017). Prevention of testicular toxicity has been considered as an important strategy to restore fertility. Studies have demonstrated the ameliorative effect of the *Moringa oleifera* and its extracts against heavy metals like mercury, chromium and cadmium (Abarikwu et al., 2017; Obembe and Raji, 2018; Sadek, 2013). We have earlier demonstrated that ethanolic extract of *Moringa oleifera* leaves mitigated CP-induced testicular toxicity by improving sperm functional characteristics and protecting the spermatogonial cells from CP-induced DNA damage (Nayak et al., 2016a, 2016b). Since the *Moringa oleifera* leaf extract is rich phytosterols (Maiyo et al., 2016) (Vergara-Jimenez et al., 2017), in the present study we wanted to see whether administering the extract during chemotherapy helps in improving endocrine function along with testicular function.

2. Materials and methods

2.1. Animals

For the experiment, male Swiss albino mice (8–10 week) were taken from inbred colony maintained in Central Animal Facility, Kasturba Medical College, Manipal Academy of Higher Education. The animals were maintained under standard conditions of temperature (25 ± 2 °C), humidity (45–55%), light (12:12 h of light and dark), food and water *ad libitum*. Institutional Animal Ethical Committee (No. IAEC/KMC/78/2011–2012) approval was obtained to carry out the experiment. All animal procedures were performed in accordance with guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Department of Animal Husbandry and Dairying, Ministry of Agriculture and Farmers Welfare, Government of India.

2.2. Preparation of extract from Moringa oleifera leaves

Information regarding the collection of leaves, plant authentication, preservation of specimen in herbarium, preparation of ethanolic extract and characterization of the extract were mentioned in our earlier studies (Nayak et al., 2016a, 2016b). Briefly, leaves were collected from the plant grown in local regions (Manipal-Udupi, South India) in the month of January–February. The plant was authenticated by Dr. Gopalakrishna Bhat, Professor and Head (Retired), Department of Botany, Poornaprajna College, Udupi, India. The specimen was preserved in the herbarium (voucher number PP610) of Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, for future reference. The leaves were shade dried, powdered and extract was prepared by Soxhlet extraction process using ethanol as solvent. The extract was characterized using high performance thin layer chromatography (HPTLC) and standardized with reference to known active compounds quercetin and chlorogenic acid.

2.3. Animal treatment

i) Effect of CP and/or MOE on endocrine profile and testis cell specific gene expression: Healthy adult male mice (8-10 week) were divided into four groups and treated as follows.
   - *Control:* Injected with PBS (phosphate buffered saline, 0.3 mL), 5 days in a week for 4 weeks
   - *CP:* Injected with CP (100 mg/kg) intraperitoneally, once in a week for 3 weeks
   - *MOE*: Injected with MOE (100 mg/kg) intraperitoneally, 5 days in a week for 4 weeks
   - *MOE + CP*: Injected with MOE (100 mg/kg, every week, for 4 weeks) 24 h prior to CP (100 mg/kg, every week for 3 weeks). At 35 day of post first CP dose, the animals were sacrificed for the various parameters.

ii) Effect of CP and/or MOE on DNA damage and repair kinetics in spermatogonial cells: Healthy prepubertal male mice (2 weeks) were divided into 4 groups and treated as follows:
   - *Control:* Injected with PBS (0.3 mL), single injection
   - *CP:* Injected with CP (200 mg/kg) intraperitoneally, single dose
   - *MOE*: Injected with MOE (100 mg/kg) intraperitoneally, single

dose

- *MOE + CP*: Injected with MOE (100 mg/kg) followed by CP (100 mg/kg), 24 h later

Animals were sacrificed at 0, 1, 2, 4, 6 and 24 h post treatment and spermatogonial germ cells were isolated.

2.4. Collection of blood, testis and epididymis

Blood was collected by cardiac puncture in a 0.5 mL micro-centrifuge tube and allowed to clot for 30 min at room temperature. The whole blood was centrifuged at 2000 rpm for 5 min at room temperature and the clear serum was transferred to another sterile micro-centrifuge tube. It was stored at −80 °C for the estimation of hormones. The testis and epididymis were collected. The tunica albuginea was removed carefully from the testis and subjected to homogenization (POLYTRON PT 1300D, Switzerland) in PBS (pH 7.4) at 7000 rpm for 5 min. The clear supernatant was obtained by centrifuging the whole homogenate at 5000 rpm for 10 min at 4 °C and stored at −80 °C until further analysis.

2.5. Assessment of functional characteristics of spermatozoa

The functional competence of spermatozoa such as density, motility, head morphology and DNA integrity were assessed by the method described in our earlier report (Nayak et al., 2016b). Briefly, sperm count was calculated using Makler chamber and expressed as millions/mL. The motility was analyzed by placing sperm suspension on the slides and counting at least total of 200 spermatozoa and categorizing them into motile and non-motile spermatozoa. The head defects and DNA damage was assessed by Shorr staining and sperm chromatin dispersion assay (SCD) respectively and expressed as percentage.

2.6. Estimation of reproductive hormones in serum and testis

Testosterone (Cat. no. EIA-1559, DRG Diagnostics, Germany), FSH (Cat. No. FS232F, Calbiotech, USA) and Inhibin B (Cat. No. EIA-INB, RayBiotech, USA) concentrations were measured by using ELISA (Enzyme linked immunosorbent assay) kits according to the manufacturer instructions.

2.7. Isolation of spermatogonial germ cells from prepubertal mouse testis

To understand DNA damage and repair kinetics in spermatogonial cells, the testicular cells were isolated from prepubertal mice by enzymatic digestion followed by differential plating as described earlier (Nayak et al., 2016a) with minor modifications. Briefly, de-capsulated testes were digested in Dulbecco's Modified Eagles Medium (DMEM, Cat. No. D5648, Sigma Aldrich, USA) containing 1 mg/mL of collagenase type IV (Cat. No. 17104–019, Gibco, USA) and 1 mg/mL of trypsin (Cat. No. RM 713, Himedia, India) at 37 °C for 20 min in a water bath with gentle mixing for every 5 min. The medium was removed to eliminate most of the interstitial cells like peritubular and Leydig cells. The seminiferous tubules were further incubated in DMEM containing collagenase type IV and trypsin for 20 min at 37 °C. Enzyme activity was neutralized by adding DMEM supplemented with 10% fetal calf serum (FCS, Cat. No. CCS-500-SA-U, Genetics Biotech Asia Pvt. Ltd., India). The cells were pelleted and seeded in a culture dish containing DMEM with 10% FCS. The somatic cells (myoid and Sertoli cells) were eliminated by incubating for 4 h in DMEM containing 10% FCS at 37 °C and 5% CO_2 where most of these cells were going attach to dish. The floating cells enriched with spermatogonial cells (germ cell fraction) were collected, washed and re-suspended in PBS and used for further analysis.

2.8. RNA extraction

The RNA from whole testis was isolated using method described earlier (Nayak et al., 2016a). Briefly, the tunica albuginea of testis was removed carefully and washed in PBS. The testis was lysed in TRI reagent (Cat. No. T9424, Sigma Aldrich, USA) by vigorous shaking and chloroform was added to the lysate. The aqueous phase was transferred to fresh tube post centrifugation at 12000 rpm for 15 min at 4 °C and isopropanol was added to precipitate the RNA. After centrifugation and washing with 70% ethanol, RNA pellet was dissolved in diethyl pyrocarbonate (DEPC) treated water. The quantity and purity were characterized using a UV spectrophotometer (Eppendorf BioPhotometer plus, Germany).

2.9. cDNA synthesis and quantitative reverse transcriptase polymerase chain reaction (qRT PCR)

First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription with a ProtoScript^R M-MuLV first-strand synthesis system kit (Cat. No. E 6300S, New England Biolabs, UK) according to the manufacturer's instructions. The relative expression of germ cell markers [Pou5f1 (Oct4), Ddx4 (Vasa) and Androgen receptor (Ar)], Sertoli cell markers [Androgen binding protein (Abp), Transferrin, Follicle stimulating hormone receptor (Fshr) and Gata4), Leydig cell markers [Steroid acute regulatory protein (Star), 17β-hydroxy steroid dehydrogenase (17β-Hsd) and Leutinizing hormone receptor (Lhr)], antioxidant enzymes [Superoxide dismutase 1 (Sod1), Catalase, Glutathione peroxidase 4 (Gpx4), Glutathione S transferase (Gst) and Glutathione reductase (Gsr)], DNA damage (P53 and Caspase3) and DNA repair [Rad51 and Xrcc5 (Ku80)] genes were estimated with reference to Glyceradehyde phosphate dehydrogenase (Gapdh) and Actin-β as housekeeping genes by Step one plus (Applied biosystems, USA) real-time PCR system using SYBR Green chemistry (Cat. No. RR420A, TaKaRa, Japan) and Taqman chemistry (Cat. No. RR390A, TaKaRa, Japan). The primer sequence and probe details were provided in Table 1. The amplification conditions were as follows: initial denaturation at 94 °C for 2 min followed by 40 cycles comprising denaturation at 94 °C for 30 s, primer annealing at 65 °C for 30 s, and extension at 72 °C for 45 s. The final extension was carried out for 7 min at 72 °C. The fluorescence emitted at each cycle was collected for the entire period of 30 s during the extension step of each cycle. The homogeneity of the PCR amplicon was determined by studying the melt curve. Mean Ct values generated and the cDNA concentrations in the samples were computed and normalized to Gapdh. The relative expression levels in terms of fold change in terms of relative quantification (RQ) values were calculated by 2 –ΔΔCt method.

2.10. Immunofluorescence

γ-H2AX and RAD51 expression in testicular germ cells were assessed by immunofluorescence. The testicular cells were fixed using 4% paraformaldehyde, permeabilized and blocked by incubating with 0.1% Triton X-100 in PBS and 10% goat serum respectively. Cells were then incubated with anti-phospho histone H2AX antibody (Cat. No. 05–636, Merck Millipore, USA) and anti-RAD51 antibody (Cat. No. ABE 257, Merck Millipore, USA) followed by incubation with goat anti-mouse IgG-FITC (Cat. No. SC-2010, Santacruz, USA) and goat anti-rabbit IgG-Alexa fluor (Cat. No. ab150077, Abcam, USA) respectively. The number of γ-H2AX and RAD51 positive cells were counted using the fluorescence microscope (Carl Zeiss, Germany) and expressed in percentage.

2.11. Statistical analysis

The data was presented as mean and standard error (Mean ± SEM). The statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison test when data obeys

Table 1
List of primers (A) and probes (B) used for quantitative reverse transcriptase PCR (qRT-PCR).

A.

Gene	Forward primer	Reverse primer
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Abp	TAGCGCATTCAATGGTACACC	TTGTGGTTCCAGTGTTTGGGA
Transeferrin	GCTGTCCCTGACAAAACGGT	TGCGGAAGGACGGTCTTCATG
Fshr	CCTTGCTCCTGGTCTCCTTG	CTCGGTCACCTTGCTATCTTG
17β-Hsd	ACTTGGCTGTTCGCCTAGC	GAGGGCATCCTTGAGTCCTG
Lhr	CTCGCCCGACTATCTCTCAC	ACGACCTCATTAAGTCCCCTG
Gata4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGT
P53	GACCGCCGTACAGAAGAAGA	GCGGATCTTGAGGGTGAAATA
Caspase3	ATGGAGAACAACAAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC

B.

Gene	Assay Id
Actin-β	Mm02619580_g1
Pou5f1 (Oct4)	Mm03053917_g1
Ddx4 (Vasa)	Mm00802445_m1
Star	Mm00441558_m1
Androgen receptor (AR)	Mm00442688_m1
Sod1	Mm01344233_g1
Catalase	Mm00437992_m1
Gpx4	Mm00515041_m1
Gst	Mm00494798_m1
Gsr	Mm00439154_m1
Rad51	Mm00487905_m1
Ku80 (Xrcc5)	Mm00550142_m1

normal distributions and Kruskal-Wallis test when did not obey Gaussian distributions using GraphPad InStat 3.0 statistical package (GraphPad Inc., version 3.06, USA).

3. Results

3.1. MOE administration ameliorated CP-induced reduction in sperm functional characteristics

CP administration significantly decreased relative testicular weight ($p < 0.01$), sperm count ($p < 0.001$) and total motility ($p < 0.001$) compared to control (Table 2). Head defects ($p < 0.01$) and DNA damage (non-significant) were elevated in CP treated mice compared to control. Administration of MOE did not cause any changes in testicular function and was similar to the control. However, in combination group, MOE administration increased the testis weight, sperm density, motility ($p < 0.05$) and, decreased spermatozoa bearing head defects ($p < 0.001$) and DNA damage ($p < 0.05$) compared to CP treated mice indicating the mitigating effect of MOE against CP-induced toxicity.

3.2. Administration of MOE improved endocrine function of testis in mice treated with CP

The serum and testicular endocrine profile are depicted in Table 3. The serum and intra-testicular concentration of testosterone was marginally decreased in CP and MOE treated group compared to control. Administration of MOE prior to CP decreased serum testosterone non-significantly without altering testicular testosterone compared to CP. These results suggest that Leydig cells are relatively resistant to CP treatment.

CP significantly increased serum ($p < 0.001$) and intra-testicular ($p < 0.001$) FSH compared to control. FSH concentration was unaltered in both serum and testis of MOE treated mice. Administration of MOE prior to CP significantly decreased serum ($p < 0.01$) and intra-testicular ($p < 0.001$) FSH levels. Further, inhibin B concentration was decreased in serum ($p < 0.05$) and testis (non-significant) of CP treated mice compared to control. MOE treatment decreased serum inhibin B but its concentration was elevated in testis compared to control. Administration of MOE with CP decreased serum inhibin B level and increased its concentration in testis.

3.3. Administration of MOE modulated expression of genes involved in testicular steroidogenesis in mice treated with CP

The expression of Star (1.1 fold) and 17β-Hsd (1.7 fold) were marginally down-regulated by CP treatment compared to control. MOE treatment also marginally down-regulated the expression of these genes compared to control. However, MOE treatment prior to CP up-regulated expression of 17β-Hsd (1.3 fold) while Star expression was down-

Table 2
Effect MOE on sperm functional characteristics in mice treated with CP at 35 day post treatment.

Treatment groups	Relative testicular weight (mg)	Sperm count (millions/mL)	Total motility (%)	Head defects (%)	DNA damage (%)
Control	342.26 ± 30.39	23.06 ± 0.84	75.83 ± 1.42	9.16 ± 0.90	10.33 ± 1.35
CP	229.66 ± 20.70[a]	5.13 ± 1.25[b]	30.83 ± 4.75[b]	21.83 ± 4.43[a]	16.66 ± 1.85
MOE	343.76 ± 19.18	20.33 ± 1.64	62.16 ± 3.89	7.83 ± 1.97	10.83 ± 1.01
MOE + CP	256.20 ± 12.38	11.88 ± 2.02	48.00 ± 1.30[c]	10.2 ± 1.52[d]	6.00 ± 1.14[c]

The values are expressed as Mean ± SEM (n = 6).
[a] $p < 0.01$.
[b] $p < 0.001$ vs Control.
[c] $p < 0.05$.
[d] $p < 0.001$ vs CP.

Table 3
Effect MOE on serum and intra-testicular reproductive hormone levels in mice treated with CP at 35 day post treatment.

Treatment groups	Testosterone (ng/mL)		FSH (mIU/mL)		Inhibin B (pg/mL)	
	Serum	Testis	Serum	Testis	Serum	Testis
Control	0.51 ± 0.02	8.59 ± 2.49	4.00 ± 0.36	8.00 ± 0.36	423.16 ± 16.46	277.33 ± 15.15
CP	0.37 ± 0.06	7.56 ± 2.69	10.50 ± 0.42[b]	13.50 ± 0.22[b]	340.00 ± 15.11[a]	195.00 ± 24.22
MOE	0.46 ± 0.23	7.37 ± 2.80	4.50 ± 0.56	7.33 ± 0.42	331.83 ± 10.70	291.16 ± 5.87
MOE + CP	0.28 ± 0.01	7.99 ± 2.95	7.83 ± 0.40[c]	10.33 ± 0.33[d]	319.00 ± 13.87	269.80 ± 8.78

The values are expressed as Mean ± SEM (n = 6).
[a]p < 0.05.
[b]p < 0.001 vs Control.
[c]p < 0.01.
[d]p < 0.001 vs CP.

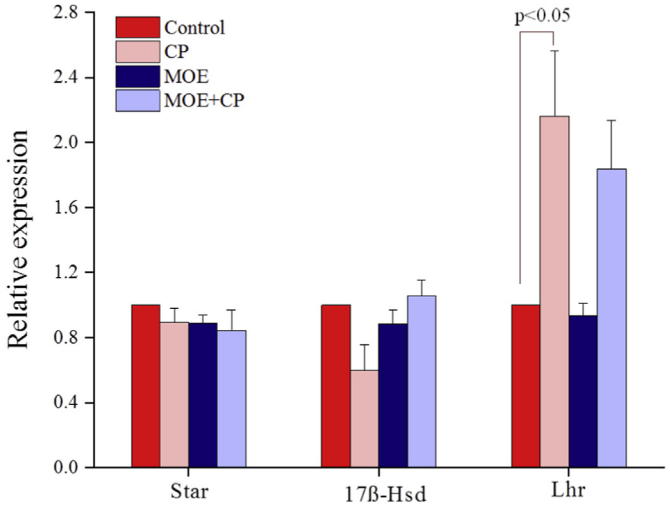

Fig. 1. Effect of MOE on the expression of steroidogenic genes in the testis of mice treated with CP. Testicular RNA was isolated at 35 day post treatment of CP, followed by cDNA synthesis and analysis of mRNA expression by qRT-PCR for the genes 17β-Hsd, Star and Lhr. Data was represented as Mean ± SEM (n = 6). [a]p < 0.05 vs Control.

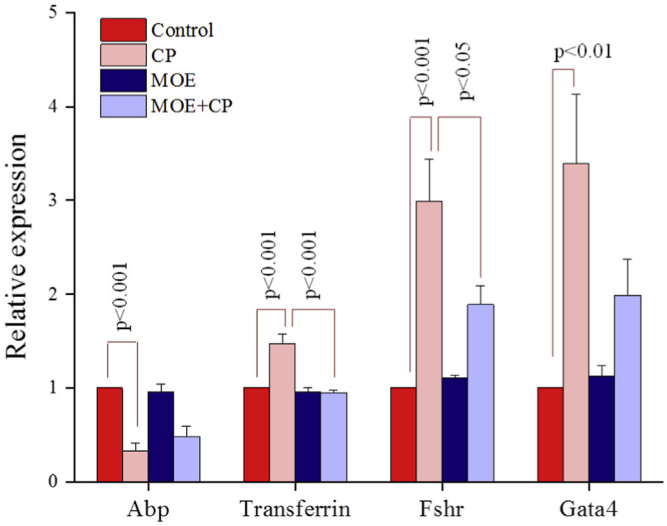

Fig. 2. Effect of MOE on the expression of genes pertaining to the Sertoli cell function in the testis of mice treated with CP. Testicular RNA was isolated at 35 day post treatment of CP, followed by cDNA synthesis and analysis of mRNA expression by qRT-PCR for the genes Abp, Transferrin, Fshr and Gata4. Data was represented as Mean ± SEM (n = 6). [a]p < 0.01, [b]p < 0.001 vs Control; [c]p < 0.05, [d]p < 0.001 vs CP.

regulated marginally compared to CP. In addition, CP administration up-regulated the expression of Lhr significantly (2 fold, p < 0.05) compared to control. Lhr expression pattern was similar in MOE and control group while administration of MOE with CP, marginally down-regulated its expression compared to CP (Fig. 1).

3.4. Sertoli cell function in testis treated with CP was modulated by MOE administration

CP treatment resulted in a significant decrease in the expression of Androgen binding protein (3.3 fold, p < 0.001) and significant increase in the expression of Transferrin (1.5 fold, p < 0.001), Fshr (3 fold, p < 0.001) and Gata4 (3.4 fold, p < 0.01) compared to control (Fig. 2). Expressions of these genes were similar in MOE treated mice compared to the control. Administration of MOE prior to CP, up-regulated expression of Abp (1.5 fold) while expression of Transferrin (1.55 fold, p < 0.001), Fshr (1.57 fold, p < 0.05) and Gata4 (1.7 fold) were down-regulated compared to CP.

3.5. MOE administration up-regulated the expression of genes related to spermatogonial cell proliferation in testis treated with CP

CP treatment down regulated expression of Oct4 and Ddx4 by 2.5 (p < 0.001) and 2.3 (p < 0.001) fold respectively compared control (Fig. 3). The expression of these two genes was not altered by MOE administration. MOE administration prior to CP, up-regulated the expression of Oct4 and Ddx4 by 2 (p < 0.05) and 2.4 fold (p < 0.001) respectively compared to CP. However, there was no change in the expression pattern of androgen receptor (Ar) in all treated groups (see Fig. 4).

3.6. CP-induced oxidative stress in testis was mitigated by MOE via modulation of expression of antioxidant enzymes

Administration of CP down-regulated the expression of antioxidant enzymes Sod1, Cat, Gpx4, Gst and Gsr. Among these Gpx4 and Gst expression were significantly down-regulated by 1.78 (p < 0.05) and 1.64 (p < 0.01) fold respectively compared to control. MOE administration did not alter the expression of these enzymes while MOE treatment with CP up-regulated expression of these antioxidant enzymes (Fig. 4).

3.7. Administration of MOE reduces DNA damage and enhances repair process in spermatogenic cells treated with CP

Immunofluorescence analysis (Fig. 5) showed that the expression of γ-H2AX reached peak at 6h after CP injection and gradually decreased thereafter. Administration of MOE to mice 24 h prior to CP showed significant decrease in the expression of γ-H2AX at all the intervals when compared to CP.

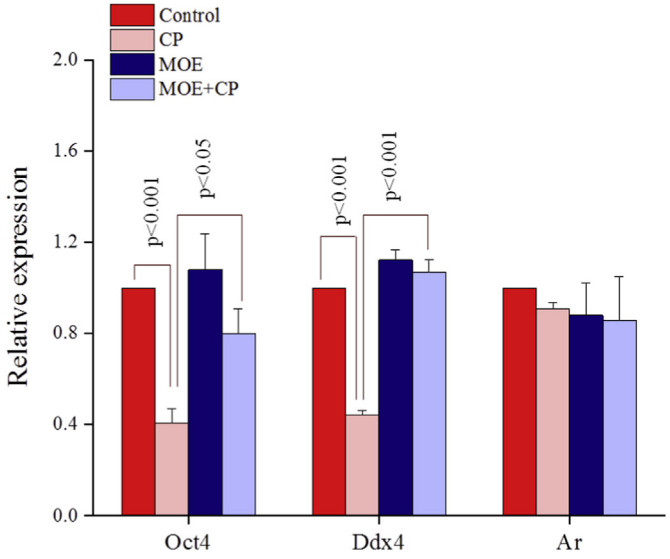

Fig. 3. Effect of MOE on the expression of genes specific to the germ cells in the testis of mice treated with CP. Testicular RNA was isolated at 35 day post treatment of CP, followed by cDNA synthesis and analysis of mRNA expression by qRT-PCR for the genes Oct4 (Pou5f1), Ddx4 (Vasa) and Ar. Data was represented as Mean ± SEM (each group n = 6). $^{a}p < 0.001$ vs Control; $^{b}p < 0.05$, $^{c}p < 0.001$ vs CP.

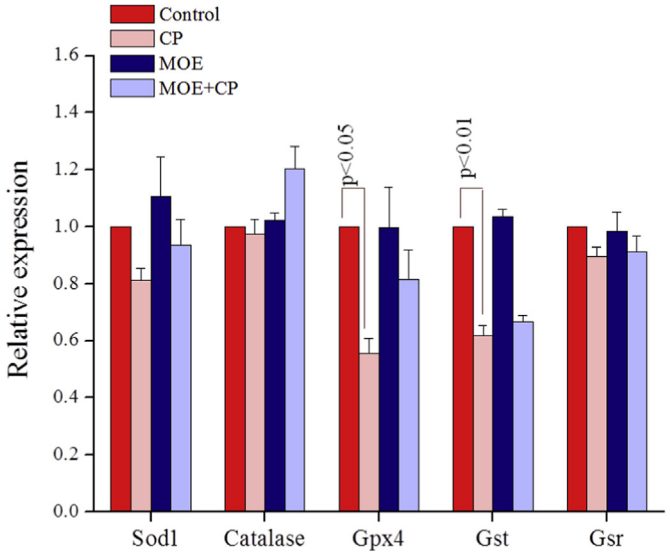

Fig. 4. Effect of MOE on the expression of genes encoding antioxidant enzymes in the testis of mice treated with CP. Testicular RNA was isolated at 35 day post treatment of CP, followed by cDNA synthesis and analysis of mRNA expression by qRT-PCR for the genes Sod1, Catalase, Gpx4, Gst and Gsr. Data was represented as Mean ± SEM (each group n = 6). $^{a}p < 0.05$, $^{b}p < 0.01$ vs Control.

The baseline expression of RAD51 in spermatogonial cells isolated from control mice was higher (25.73 ± 4.14) indicating the homologous recombination during meiosis. RAD51 expression was elevated post CP treatment by 1.3 fold compared to control at 24 h. RAD51 positive cells were marginally higher in MOE treated spermatogonial cells. However, administration of MOE prior to CP, decreased the expression of RAD51 by 1.28 fold compared to CP (Fig. 6).

3.8. MOE administration modulated expression of genes related to DNA damage and repair in spermatogonial cells exposed to CP

CP administration resulted in up-regulation of P53 (1.6 fold) and Caspase3 (1.18) expression at 24 h compared to control. MOE administration showed similar expression pattern for these two genes as that of control. Administration of MOE prior to CP resulted in decreased expression of P53 and Caspase3 compared to CP (Fig. 7).

The analysis of mRNA expression of Rad51 and Ku80 (DNA repair proteins involved in non-homologous end joining repair) showed down-regulation of these two genes in CP treated spermatogonial cells at 24 h compared to control (Fig. 7). MOE administration marginally decreased the expression of these two genes compared to control. Administration of MOE prior to CP did not alter the expression of Rad51 while Ku80 expression compared to CP.

4. Discussion

Testicular toxicity is the major long term consequence of cancer therapy and there is a need for a practically feasible method to preserve the fertility in pre-pubertal boys. The earlier studies conducted by our group have demonstrated (Nayak et al., 2016a, 2016b) that administration of ethanolic extract of *Moringa oleifera* effectively mitigated CP-induced testicular damage by improving sperm functional competence and proliferation of spermatogonial cells. The present study was planned to understand whether MOE mediates chemoprotection against CP through improving endocrine and cell specific function of testicular microenvironment which are the key factors in maintaining normal spermatogenesis. The results of the present study demonstrated that MOE mitigated CP-induced testicular toxicity by improving endocrine profile and modulating expression of genes specific for spermatogonial and Sertoli cell function. The downregulation of γ-H2AX expression caused by administration of MOE 24 h prior to CP at different time interval suggests the reduction in double DNA strand breaks induced by CP.

Testosterone is a key androgen which plays a vital role in maintenance of blood-testes barrier integrity, meiosis, Sertoli-spermatid adhesion and release of spermatozoa to the lumen (Smith and Walker, 2014). We observed marginal decline in serum and intra-testicular testosterone after CP administration suggesting that Leydig cells are relatively resistant to chemotherapy-induced damage. Our observation agrees with earlier reports which demonstrated that testosterone level is either unaltered or decreased to subnormal level post chemotherapy (Berger et al., 1996; Gerl et al., 2001; Howell et al., 1999). Interestingly, testosterone level was decreased in MOE alone group. The possible reason underpinning this observation could be due to the presence of phytosterols in extract which was observed in our preliminary screening. In support of this finding, an earlier study demonstrated that phytosterols reduce testosterone production in testis of male Japanese quail by modulating gonadotropin inhibitory hormone (GnIH) and gonadotropin releasing hormone GnRH (Qasimi et al., 2018). Furthermore, study on rats have shown that phytosterols decreases the activity of the testosterone metabolizing enzymes (Awad and Fink, 2000). These phytosterols has also been shown to decrease low density lipoprotein (LDL) cholesterol which serves as a source of cholesterol for the biosynthesis of testosterone and other steroid hormones (Ras et al., 2014).

FSH and inhibin B level in serum can be used as markers to assess degree of testicular damage (Islam and Trainer, 1998; Pierik et al., 2003). In this study, we observed that CP treatment significantly altered the levels of FSH and inhibin B. These findings are in accordance with earlier studies where, chemotherapeutic regimen for hematological malignancy resulted in elevated FSH and LH and declined Inhibin B (Bordallo et al., 2004; Wallace et al., 1997). Cumulative doses of cyclophophamide and procarbazine resulted in increased FSH and decreased inhibin B in long term cancer survivors (van Casteren et al., 2009) indicating that these drugs have a prolonged effect. The elevation

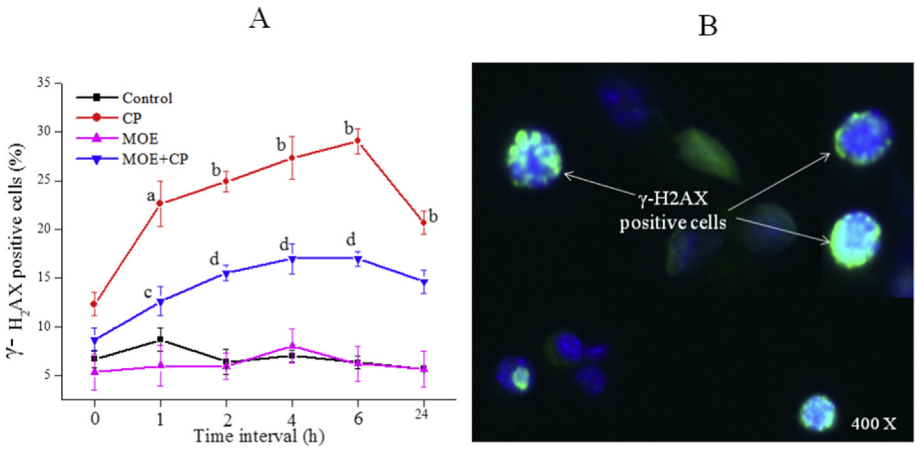

Fig. 5. Effect of MOE on the expression of γ-H2AX in the testis treated with CP at 0, 1, 2, 4, 6 and 24 h interval. The isolated spermatogonial cells were subjected immunofluorescence using anti-phospho histone H2AX antibody followed by goat anti-mouse IgG-FITC and counterstaining nucleus with DAPI. Data was represented as Mean ± SEM (n = 4). $^{a}p < 0.01$, $^{b}p < 0.001$ vs Control; $^{c}p < 0.05$, $^{d}p < 0.01$ vs CP.

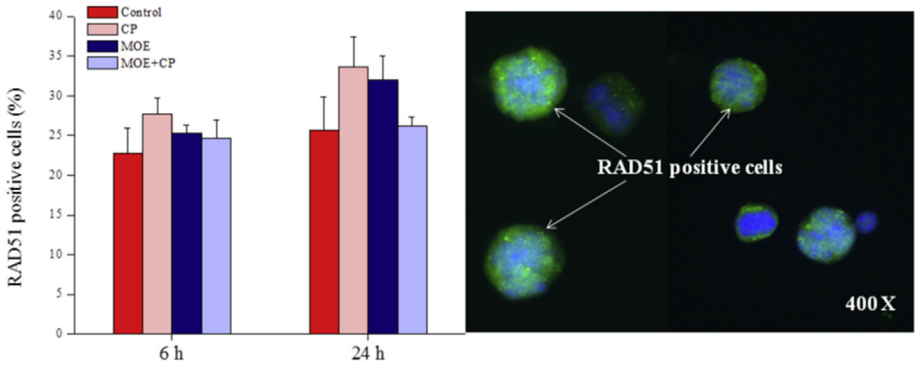

Fig. 6. Effect of MOE on the expression of RAD51 in the testis of mice treated with CP at 6 and 24 h time interval. The isolated spermatogonial cells were subjected immunofluorescence using anti-RAD51 antibody followed by goat anti-rabbit IgG-Alexa fluor and counterstaining nucleus with DAPI. Data was represented as Mean ± SEM (n = 4).

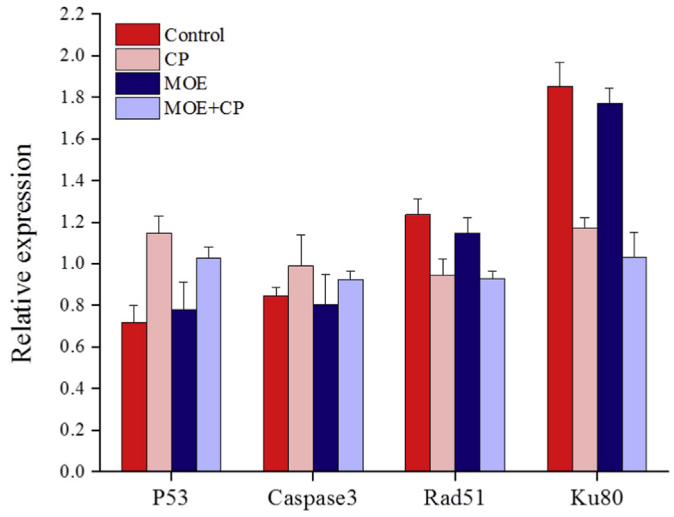

Fig. 7. Effect of MOE on the expression of P53, Caspase3, Rad51 and Ku80 (Xrcc5) in spermatogenic cells of mice treated with CP at 24 h interval. RNA was isolated from spermatogonial cells at 35 day post treatment of CP, followed by cDNA synthesis and analysis of mRNA expression by qRT-PCR. Data was represented as Mean ± SEM (each group n = 3).

of FSH during testicular damage could be an adaptive response to germ cell depletion or seminiferous tubule damage and decreased level of inhibin B suggesting the dysfunction of Sertoli cell due to CP-induced toxicity. The level of FSH and Inhibin B was reverted by administration of MOE along with CP indicating alleviation in germ cell depletion and Sertoli cell toxicity. Marginal change in the expression of cholesterol transport protein Star and steroidogenesis enzyme 17β-Hsd observed in this study further supports the unaltered testosterone concentration post CP treatment. It has been shown that anticancer regimen involving bleomycin, etoposide and cisplatin, decreased the expression of 17β-Hsd without altering expression of Star (Al-Bader and Kilarkaje, 2015). However, we observed higher expression of Lhr following CP treatment which could be due to defect in feedback regulation. A similar finding has been observed earlier where radiation causes increase in Lhr expression (Sivakumar et al., 2006).

Sertoli cells support spermatogenesis by nurturing the proliferating germ cells by secreting wide array of compounds like growth factors, cytokines and transport proteins. Transferrin and androgen binding protein are two functional markers which are regulated by FSH (Skinner et al., 1989). We observed that CP treatment increased in expression of Fshr and transferrin whereas expression of Abp was decreased. Earlier reports have shown that ABP concentration in blood is correlated to Sertoli cell number (Orth et al., 1988). The biphasic change in ABP level was observed in blood and testis post busulfan treatment (Morris et al., 1987) where in early germ cell depletion showed decreased and increased level of ABP in serum and testis respectively. While late germ cell depletion resulted in increased serum and decreased testis ABP level. In the present study, Abp mRNA abundance was decreased at 35 day post treatment of CP indicating depletion of late spermatogenic cells. The up-regulated expression of Fshr might be due to increased FSH level. The previous studies have shown that FSH can stimulate the transferrin production (Chaudhary et al., 1996; Skinner et al., 1989). It was reported that over expression of transferrin affected testicular function in aged mice (Lecureuil et al., 2007). Overall these alteration induced by anticancer agents affect the germ cell proliferation and differentiation.

The anticancer agents perturb germ cell proliferation by two ways. The direct effect is by inducing DNA damage (Nayak et al., 2016a; Stukenborg et al., 2018) or indirect way is via affecting the secretion of hormones, growth factors and other molecules secreted by somatic cells like Leydig and Sertoli cells as observed in the present study. CP induces DNA damage and apoptosis in germ cells which results in depletion of

spermatogenic cells in testes (Nayak et al., 2016a). Here, we observed the decreased the expression of Oct4 (maker of undifferentiated SSC) and Ddx4 (marker of differentiated SSC) post CP administration which indicate the loss of germ cell population.

Oxidative stress is one of the major consequences of CP-induced testicular toxicity by generating free radicals and suppressing antioxidant defense system. In our previous study we observed increased lipid peroxidation and decreased activity of SOD and Catalase (Nayak et al., 2016b). Here we studied the expression of antioxidant enzymes at mRNA level. Among these, Gpx4 (testis specific) and Gst were down-regulated significantly while expression of other genes was marginally decreased by CP administration. MOE has shown to possess tremendous antioxidant property by scavenging reactive oxygen species which is attributed to the presence of antioxidant molecules such as vitamin C, vitamin K and polyphenols (Atawodi et al., 2010; Verma et al., 2009). It has been shown that leaf extract in mouse can restore GSH and prevent lipid peroxidation induced by radiation in liver (Sinha et al., 2011, 2012). Moreover, administration of hydroethanolic extract of leaves in rats attenuated paracetamol induced hepatotoxicity by decreasing lipid peroxidation and restoring the levels of GPX, GST and GR (Uma et al., 2010).

CP is known for inducing DNA damage in cells by alkylation and generating reactive oxygen species. In response to DNA damage a cell can activate signaling events to correct the damage. Phosphorylation of histone (γ-H2AX) is one of the initial event during DNA damage (Fernandez-Capetillo et al., 2004) which serves as a marker of DNA double strand break (Rogakou et al., 1998; Takahashi and Ohnishi, 2005). Appearance and disappearance of γ-H2AX foci is an indicator of dynamics of DNA damage and repair induced by clastogenic agent like radiation (Mariotti et al., 2013; Paris et al., 2011). We studied the kinetics of γ-H2AX post CP injection in spermatogenic cells which was increased compared to all other groups and reached to peak at 6 h. Co-administration of MOE with CP decreased γ-H2AX level indicating prevention of DNA damage. It has been shown that chlorogenic acid reduces DNA damage induced by isoproternol by decreasing expression of γ-H2AX, ataxia telangiectasia mutated (ATM), breast cancer antigen 1 (BRCA1) in smooth muscle cells (Wang et al., 2016). Further studies on poly-phenols of plant origin have shown to decreased DNA damage induced by external toxicants and also enhance repair process (Azqueta and Collins, 2016).

DNA repair process helps cell to overcome endogenous and exogenous insults which are essential for maintaining genomic integrity. Rad51 is a double strand break repair protein through homologous recombination and exhibits DNA-dependent ATPase activity. It is involved in repair of radiation and alkylating agent-induced DNA breaks. It has been shown that increased expression of DNA repair genes in cancer cell related to poor prognosis and tumor resistance (Alshareeda et al., 2016). We observed increase in the expression of RAD51 in CP treated testicular cells which are probably due to the attempt of cells in repairing DNA double strand break induced by CP. However, m-RNA abundance for Rad51 and Ku80 was decreased and the possible reason might be the alkylation mRNA species by CP and subsequent degradation. Administration of MOE marginally decreased expression of Rad51 and this observation is in accordance with earlier study where natural compounds like resveratrol has shown to decrease the expression of RAD51 in cancer cells, interfere with repair process and increases sensitivity of anticancer drugs (Leon-Galicia et al., 2018). In our earlier study, we have demonstrated high concentration of phenolic compounds such as quercitin and chlorogenic acid (Nayak et al., 2016a) in MOE and these compounds are known to exhibit wide range of biological activity. It has been shown that quercetin protects DNA by decreasing the damage and enhancing repair by modulation of DNA repair enzymes (Min and Ebeler, 2009). An *in vitro* study showed that DNA protecting effect of flavonoids is concentration-dependent. At lower doses, flavonoids protects the DNA from hydrogen peroxide (H_2O_2) while, at higher concentration it induces damage and apoptosis (Watjen et al., 2005). The presence of these compounds along with wide array of other phytochemicals in MOE could potentially exhibit modulation of multiple signaling pathways that regulates cell cycle, metabolism, immune system and apoptosis. In future, understanding these events in biological system could provide a strong evidence for the prevention of toxicity induced by various gonadotoxic agents on normal cells by natural products and this would be relatively safe and cost-effective approach in maintain general health condition.

5. Conclusions

In conclusion, the present study demonstrated that MOE can mitigate CP-induced testicular dysfunction by improving blood and intratesticular hormone milieu (Testosterone, FSH and inhibin B) which is a key factor in maintaining spermatogenesis. The modulation in the expression pattern of genes pertaining Sertoli cells (Abp, Tranferrin, Fshr, Gata4), spermatogenic cells (Oct4 and Ddx4) and antioxidant system (Sod1, Catalase, Gpx4, Gst and Gsr) reflects the protective action of MOE. Further, the resistance of Leydig cell to CP-mediated insults was quite evident from the marginal changes in testosterone level as well as expression of genes involved in steroidogenesis. Modulation of kinetics of DNA damage (γ-H2AX) and repair (RAD51) in germ cells suggests the chemoprotective role of MOE which could serve as promising method of fertility preservation in pre-pubertal boys undergoing chemotherapy in future. However, further studies are required to evaluate differential protective action of MOE in two distinct cell types such as testicular and cancer cells by using appropriate tumor model prior to its use in clinical set up.

Contributors

GN, AR, PM, SM performed experiments and collected the data; SM and SGK helped in statistical evaluation of the data; SKA helped in manuscript writing; GK designed the study and wrote the manuscript.

Declaration of competing interest

The Authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112922.

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