Journal of Ethnopharmacology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Effect of standardized extract of *Bacopa monnieri* (CDRI-08) on testicular functions in adult male mice

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ARTICLE INFO

Article history:
Received 25 December 2015
Received in revised form
21 June 2016
Accepted 8 July 2016

Keywords:
Bacopa monnieri (CDRI-08)
Germ cell proliferation
Spermatogenesis
Steroidogenesis
Spermatozoa

ABSTRACT

Ethnopharmacological relevance: *Bacopa monnieri* (BM) has been used in India since the time of *Rig-Veda* for augmentation of learning, memory, brain health etc.
Aim of the study: The memory augmenting effect of BM is well documented. CDRI-08 is a standardized extract of *Bacopa monnieri*, but its effect on the male reproductive health has not been investigated. Therefore, the aim of the present study was to examine the effect of CDRI-08 administration on the male reproductive organs with special emphasis on testis in adult mice.
Materials and methods: CDRI-08, containing at least 55% bacosides (the major constituent of BM), was investigated for its effect on testicular functions in adult Parkes (P) mice. A suspension of CDRI-08 was orally administered in doses of 40 and 80 mg kg^{-1} body weight day^{-1} for 28 days and various male reproductive end points were evaluated.
Results: Compared to control, CDRI-08 treatment caused a significant increase ($p < 0.05$) in spermatogenic cell density (germinal epithelial height: control, 55.03 ± 4.22 vs 40 mg, 67.15 ± 2.65 and 80 mg, 69.93 ± 3.76; and tubular diameter: control, 206.55 ± 2.62 vs 80 mg, 253.23 ± 12.19), PCNA index (control, 59.85 ± 2.09 vs 40 mg, 82.17 ± 1.56 and 80 mg, 84.05 ± 3.51) and in steroidogenic indices in the testis, and in sperm viability (control, 0.67 ± 0.010 vs 80 mg, 0.80 ± 0.04) in cauda epididymidis of the treated mice. On the other hand, however, the same treatment caused a significant decrease ($p < 0.05$) in abnormal sperm morphology (control, 21.72 ± 1.06 vs 40 mg, 10.63 ± 1.50 and 80 mg, 15.86 ± 0.87) in cauda epididymidis, and in lipid peroxidation level in testis of the treated mice compared to controls.
Conclusion: The results suggest that treatment with CDRI-08 extract improves sperm quality, and spermatogenic cell density and steroidogenic indices in the testis of P mice.

1. Introduction

From the time immemorial, medicinal plants have been widely used in the treatment of various disorders and they have made significant contribution to human health. One such plant is *Bacopa monnieri*, which has been extensively investigated for its various medicinal properties (Mathur et al., 2016). Plants have also been used as supplements to improve male sexual functions (Chaturapanich et al., 2008).

Bacopa monnieri (L.) Wettst. (family, *Plantaginaceae*) known commonly as Brahmi, is a perennial, creeping herb native to wet lands in India, especially the north-east and the southern regions. CDRI-08 is a standardized extract of *Bacopa monnieri* which contains not less than 55% bacosides with an optimum concentration of bacogenins (Kean et al., 2015).

Procreation is an important moral and religious duty in human civilization. However, a large number of human population is still suffering from infertility and it is reported that 50% of infertile couples have male factors-related abnormalities (Shefi and Turek, 2006 cited in Abdillahi and Van Staden, 2012). Further, sperm abnormalities such as lack of sperm, too little sperm, abnormal sperm morphology and insufficient sperm motility may contribute to infertility (Feng, 2003 cited in Abdillahi and Van Staden, 2012). Pilot studies conducted by Jothianandan, a research scientist in the laboratory of Robert F. Furchgott (who won the Nobel Prize for Medicine and Physiology in 1998 jointly with Louis J. Ignarro and Fesid Murad) showed that the CDRI-08 activates modulation of vascular responses by endothelium-derived relaxing factors now known as nitric oxide (NO); further, this effect is similar to sildenafil citrate and it has got a mild inhibitory effect on phosphodiesterase (PDE_5) inhibition in penile erection (Jothianandan, 1998; personal communication). However, to the best of our knowledge, there is no study of the effect of CDRI-08 on the male reproductive health. The present study, therefore, examined the

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http://dx.doi.org/10.1016/j.jep.2016.07.026
0378-8741/

effect of CDRI-08 on the male reproductive organs with special focus on testis in Parkes strain mice, which we have been using for an animal model (Verma and Singh, 2016). We have assessed several male reproductive end points such as sperm parameters, histology, testosterone assay, lipid peroxidation level, immunoblot and immunohistochemical analyses of proliferating cell nuclear antigen (PCNA), and immunoblot analyses of Caspase-3, steroidogenic acute regulatory (StAR) protein, P450 side chain cleavage (P450scc) enzyme, and 3β- and 17β-hydroxysteroid dehydrogenases (3β- and 17β-HSD).

2. Materials and methods

2.1. Plant material

The specialized extract of Brahmi (CDRI-08, batch number: C15030294) was a gift from Lumen Marketing Company, Chennai, India. CDRI-08 was prepared from pulverized Bacopa raw material using ethanol extraction with not less than 55% total bacosides by spectrophotometric method of analysis. The product is brown color powder containing not less than 90% ethanolic soluble extractives, and loss on drying is not more than 10%. The extraction of CDRI-08 was done by a company, Chemiloids, Vijaywada, India and the manufacturing was done by Lumen Marketing Company, Chennai, India.

2.2. Chemicals and reagents

All the chemicals used in the present investigation were of analytical grade and purchased from HiMedia Laboratories and E. Merck India Ltd., Mumbai, unless stated otherwise. The source of the antibodies used in the present study is mentioned wherever necessary.

2.3. Animal care and maintenance

Adult (age 12–14 weeks) male mice belonging to the Parkes strain, weighing 30–35 g, were used in the present investigation. Animals were housed under hygienic conditions in a well-ventilated room at 23 ± 2 °C with a 12 h photoperiod and a relative humidity of 50 ± 20%, and they were given pellet food (Amrut Laboratory Animal Feeds, Pune, India) and drinking water ad libitum. Each group of animals was housed separately in polypropylene cages (450 mm × 270 mm × 150 mm), with dry rice husk as bedding material. General health and body weight of the animals were monitored regularly throughout the treatment tenure. Animals were maintained in the animal house of the Department of Zoology, Banaras Hindu University, and upkeep and use were according to the guidelines of the Animal Ethics Committee of the Banaras Hindu University as per approval of committee for the purpose for control and supervision of experimental animals (CPCSEA), Government of India (No. 1802/G0/Re/S/15/CPCSEA).

2.4. Experimental design and dose preparation

Animals were randomly allocated to three groups (I-III), each comprising five individuals (n=5) and treated as follows: Group I, vehicle-treated controls; Groups II and III, administration of CDRI-08, 40 mg kg^{-1} body weight day^{-1} and 80 mg kg^{-1} body weight day^{-1}, respectively, for 28 days. Doses of CDRI-08 were selected based on a pilot study performed by us in P mice. Doses were administered orally with the help of an oral feeding needle. Mice in control group received equivalent volume of vehicle (20% gum acacia in 2% Tween-20, 5 ml kg^{-1} body weight day^{-1}). The LD50 was determined by both oral and ip routes in rats and mice and it was found to be >3 g kg^{-1} body weight by oral route in both the species (Singh, 2013).

CDRI-08 extract was suspended in vehicle (20% gum acacia in 2% tween-20) and stored at 4 °C. Suspension was vortexed every time before administration.

2.5. Sample collection

After 24 h of the last treatment, final body weights of animals were recorded, and they were then sacrificed by decapitation under mild ether anaesthesia. Blood was collected and serum was separated and stored at −20 °C until further use. For histological and immunohistochemical studies, testes and epididymis were randomly excised from either the left or the right side of each mouse in a group. The testes from the other side of these mice were excised, blotted free of blood, weighed and kept frozen at −20 °C until further use. The epididymis was used for evaluation of sperm parameters. Seminal vesicle was also dissected out, blotted free of blood and weighed.

2.6. Sperm analyses

The number, motility, viability and morphology of spermatozoa were assessed. For this, cauda epididymidis of each mouse in control and treated groups was placed in a 35-mm plastic petridish containing 0.5 ml of F12 medium supplemented with 0.1%, w/v bovine serum albumin (BSA) and maintained at 37 °C for 10 min (Sarkar et al., 2015). The tissue was minced carefully in the medium with the help of fine forceps and scissors to ensure the extrusion of spermatozoa. The tissue fraction was removed with the help of fine forceps and a needle, and the suspension was used for the assessment of number, motility, viability and morphology of spermatozoa.

2.6.1. Sperm count

The number of spermatozoa was counted according to World Health Organization (1999) laboratory manual.

2.6.2. Sperm motility

Sperm motility was assessed subjectively.

2.6.3. Sperm morphology

For assessment of sperm morphology, sperm smear was prepared on a clean glass slide and observed under a phase contrast microscope. The criteria of Wyrobek and Bruce (1975) and Zaneveld and Polakoski (1977) were used for the evaluation of sperm abnormalities.

2.6.4. Sperm viability

Sperm viability was evaluated by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sarkar et al., 2015). Briefly, after sperm count, the sperm suspension was immediately diluted to get a concentration of 4×10^6 spermatozoa/ml. In each well of the 96-well microplate, 100 µl of diluted sperm suspension and 10 µl of MTT stock solution (5 mg/ml of MTT in PBS) were placed. This was performed in duplicate. Plate was incubated at 37 °C with 5% CO_2 for 1 h. Then 100 µl of acidified propanol (0.04 mol/L HCl in isopropanol) was added in each well to dissolve the formazan. After 30 min, optical density (OD) was measured on a microplate reader (ELx-800, Biotek Instruments, Winooski VT, USA) at a wavelength of 570 nm. Mean OD values of each collection of duplicates were used for statistical analyses.

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2.7. Histopathology

For histological study, testis and epididymis were fixed in Bouin's solution, dehydrated in graded ethanol series, cleared in benzene and embedded in paraffin. Tissues were sectioned at 6 μm, and the sections were stained with periodic acid Schiff (PAS) and counterstained with haematoxylin. The stained sections were then examined under a Leitz (Germany) light microscope.

2.7.1. Testis

Identification of stages of spermatogenesis in mouse testis was performed according to the criteria of Russell et al. (1990). The diameter of the seminiferous tubules and the height of the germinal epithelium were measured in stage VII round or slightly oblique seminiferous tubules (n = 10) (Verma and Singh, 2014).

2.7.2. Epididymis

Epididymis was divided into five (I-V) segments for histological observations (Mishra and Singh, 2005).

2.8. Testosterone assay

The serum level of testosterone was measured by ELISA using a commercial kit (DiaMetra, Segrate, Italy) as per manufacturer's instruction. The sensitivity of the assay was 5 pg/ml with intra- and inter-assay coefficient of variations being 5.1% and 7.5%, respectively.

2.9. Lipid peroxidation

The level of lipid peroxidation (LPO) was determined by the method of Ohkawa et al. (1979). Briefly, testis was homogenized in ice cold phosphate buffer (50 mM, pH 7.4). The homogenate was centrifuged at 2500 rpm for 10 min at 4 °C. An aliquot of the supernatant was used for LPO assay. For this, 3.3 ml thiobarbituric acid (TBA) reagent (0.2 ml of 8.0% SDS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% aqueous solution of TBA and 0.1% of butylated hydroxyl toluene) was mixed with 0.2 ml tissue supernatant and the mixture was boiled at 95 °C in a water bath for 60 min. The solution was then cooled and centrifuged at 2000 rpm for 10 min. The supernatant was then used for recording absorbance against blank (distilled water) at 532 nm.

2.10. Immunoblot analysis of testicular proteins

Testes were pooled together to prepare 10% (w/v) homogenate in lysis buffer. The homogenate was then centrifuged at 11,303 rpm for 25 min. The supernatant was collected and protein concentration was measured by Lowry's method (Lowry et al., 1951). Equal amount of protein (20–40 μg) from each group was resolved on 12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane (Merck Millipore, Bangalore, India) for overnight and blocked in 5% nonfat powdered milk in Tris buffered saline (TBS) for 2 h at room temperature. The membranes were then incubated with primary antibodies against StAR (rabbit polyclonal, 1:1000; Santa Cruz Biotechnology, Inc, CA, USA), CYP11A1 (rabbit polyclonal, 1:500; MyBiosource Inc, San Diego, CA USA), 3β-HSD (goat polyclonal, 1:500; Santa Cruz Biotechnology, Inc, CA, USA), 17β-HSD (rabbit polyclonal, 1:750; Santa Cruz Biotechnology, Inc, CA, USA), PCNA (mouse monoclonal, 1:2000; Santa Cruz Biotechnology, Inc, CA, USA), caspase-3 (rabbit polyclonal, 1:500; Thermo Fisher Scientific, Rockford, IL, USA) and α-tubulin (mouse monoclonal, 1:5000; Santa Cruz Biotechnology, Inc, CA, USA) for overnight at 4 °C. Membrane was then washed in Tris buffered saline with 0.1% Tween-20 (TBST) thrice for 10 min each, and incubated with horseradish peroxidase-conjugated secondary antibodies: goat anti-rabbit IgG (1:1000, Genei, Banglore, India) for StAR, CYP11A1, 17β-HSD and caspase-3; goat anti mouse IgG (1:5000) for PCNA and α-tubulin; and donkey anti-goat (1:5000, Santa Cruz Biotechnology, Inc, CA, USA) for 3β-HSD. Antibodies were diluted in TBS containing 2% (w/v) nonfat dry milk powder. The membranes were then washed for three times (15 min each) in TBST and the signals were detected using chemiluminescence reagents (ECL kit, Thermo Pierce, Rockford, IL, USA) on X-ray film. Blot for each protein was repeated thrice. The resulting immunoreactive bands were quantified by Image J software (NIH, Bethesda, USA).

2.11. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA)

For immunohistochemical detection of PCNA, testicular sections (6 μm-thick) were deparaffinized, rehydrated in alcohol series and washed twice in phosphate buffered saline (PBS), pH 7.4 for 10 min each. Thereafter, antigens were unmasked by microwave heating (750 W) in 10 mM citrate buffer (pH 6.0) for 10 min. After washing in PBS thrice for 5 min each, slides were incubated in 3% H_2O_2 in methanol for 20 min at room temperature to block the endogenous peroxidase activity. Non-specific binding of antibodies was blocked by incubation with 5% normal goat serum (NGS) diluted in PBS for 2 h at room temperature. The samples were incubated overnight in a humidified chamber at 4 °C with the primary antibody (mouse monoclonal PCNA antibody) at a dilution of 1:300 with 2% NGS in PBS. The sections were then washed three times (10 min each) in PBS and incubated with HRP-conjugated goat anti-mouse IgG secondary antibody for 2 h at room temperature. Following washing, sections were incubated with a solution of 3,3 diaminobenzidine tetrahydrochloride (DAB) substrate (Vector Laboratories, Burlingame, CA, USA) in dark for 5–10 min. Sections were then dehydrated through graded series of ethanol, cleared in xylene and mounted with DPX. Negative control slides were also used in this experiment. In negative control slides, instead of primary antibody, 2% NGS in PBS was added and the other steps to prepare negative control slides were the same as mentioned above. The images were viewed and analyzed under a Leitz (Germany) light microscope equipped with digital camera (Leica EC3, Germany). The PCNA-positive cells in the sections were counted in 10 tubular cross sections per testis from each mouse in a group. Proliferation index was calculated using the formula: (PCNA-positive cells/total number of germ cells in a tubule) X 100 (Sarkar et al., 2015).

2.12. Statistical analyses

All data, except for body weight, were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls' multiple range test for comparison of group means. Body weight data, however, were analyzed by Student's *t* test. Differences were considered significant at $p < 0.05$. Data were expressed as mean ± S.E.M.

3. Results

3.1. Body and sex organ weights

No significant differences were noted between the initial and final body weights of CDRI-08-treated mice and controls (Table 1). The weights of testis, epididymis and seminal vesicle were also not altered in CDRI-08-treated mice compared to controls (Table 1). Further, all the animals maintained a healthy appearance throughout the period of investigation.

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Table 1
Body weight and weights of the testis, epididymis and seminal vesicle in mice after CDRI-08 treatment.

Groups	Body weight (g)		Sex organs weight (mg)[a]		
	Initial	Final	Testis	Epididymis	Seminal vesicle
Group I	32.80 ± 1.85	34 ± 0.59	80.14 ± 6.89	45.32 ± 1.41	115.8 ± 9.12
Group II	32.40 ± 0.75	35 ± 0.47	81.80 ± 2.51	46.92 ± 0.94	118.6 ± 8.20
Group III	32.08 ± 0.97	35.4 ± 0.53	84.42 ± 6.28	45.95 ± 0.85	115.2 ± 11.49

Values are mean ± S.E.M. for five animals.

[a] Weight refers to the real weight of the unpaired organs in the animal.

Table 2
Motility, number and abnormal morphology of spermatozoa in cauda epididymidis of mice after CDRI-08 treatment.

	Group I	Group II	Group III
Motility (%)	72.80 ± 3.17	74.20 ± 1.5	74.00 ± 1.92
Number ($\times 10^6$)	15.19 ± 0.99	14.85 ± 1.13	14.65 ± 0.05
Abnormal morphology (%)	21.72 ± 1.06	10.63 ± 1.5[a]	15.86 ± 0.87[a]
Atypical head-shape (%)	5.12 ± 0.67	1.90 ± 0.33[a]	1.65 ± 0.62[a]
Flagellar defects (%)	16.67 ± 1.05	8.97 ± 1.71[a]	14.46 ± 1.23

Values are mean ± S.E.M. for five animals.

[a] Significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

3.2. Sperm analyses

No significant differences were found in the number and motility of spermatozoa in the cauda epididymidis of CDRI-08-treated mice compared to controls (Table 2). However, a significant increase was recorded in viability of spermatozoa in mice treated with 80 mg dose of the extract compared to controls (Fig. 1). Further, a significant reduction in the number of morphologically abnormal spermatozoa was noted in both dose groups of CDRI-08-treated mice compared to controls (Table 2). Among morphological abnormalities observed in spermatozoa, atypical head-shape abnormality (oval head, head without hook, head folded on tail, amorphous head and banana-shaped head) was significantly reduced in mice treated with both the doses of CDRI-08, while those with flagellar defects were reduced only in lower dose group of treated mice compared to controls (Table 2).

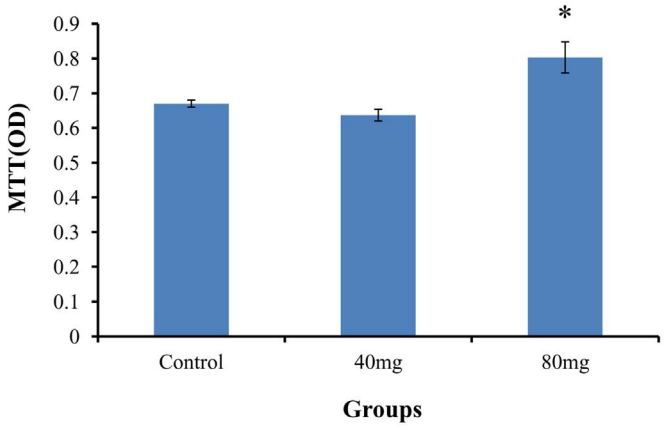

Fig. 1. Effect of CDRI-08 administration (40 and 80 mg kg^{-1} body weight for 28 days) on sperm viability by MTT assay. A significant increase in sperm viability is seen in 80 mg dose group of mice compared to controls. Data are presented as mean ± S.E.M. (n = 5). *Significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

3.3. Histopathology

3.3.1. Testis

Histologically, testis in CDRI-08-treated mice (Fig. 2B–E, C–F) showed normal features, which were similar to that of controls (Fig. 2A–D). When quantitatively analyzed, a significant increase in the height of the germinal epithelium was noted in testis of mice treated with both the doses of CDRI-08 compared to controls; the tubular diameter, however, was significantly increased only in those treated with higher dose of the extract compared to controls, while no change was recorded in lower dose group of treated mice (Table 3).

3.3.2. Epididymis

The epididymis in CDRI-08-treated mice showed normal histologic features in all the segments, which were similar to that seen in controls (photographs not shown).

3.4. Serum level of testosterone

No significant differences were found in serum level of testosterone in CDRI-08-treated mice compared to controls (Table 3).

3.5. Lipid peroxidation level

Testicular lipid peroxidation level was significantly reduced in CDRI-08-treated mice compared to controls (Table 3).

3.6. Immunoblot analysis of testicular proteins

The effect of CDRI-08 treatment on expressions of testicular StAR protein and enzymes involved in testosterone biosynthesis are shown in Fig. 3. The expressions of PCNA (a proliferation marker) and caspase-3 (an apoptosis marker) are depicted in Fig. 5. Immunoblot analyses revealed a significant increase in the relative expression of StAR protein, CYP11A1, 3β-HSD and 17β-HSD in testes of mice treated with 80 mg dose of CDRI-08 compared to controls; at 40 mg dose, there was a significant increase only in CYP11A1 and 17β-HSD compared to controls (Fig. 3). A significant increase was noted in expression of PCNA in mice treated with 80 mg dose of CDRI-08 compared to controls, while no such change was recorded in 40 mg dose group (Fig. 5C). However, no significant differences were found in expression of caspase-3 in testes of treated mice compared to controls (Fig. 5B).

3.7. Evaluation of immunohistochemical staining of PCNA

In testicular sections, immunoreactive signals of PCNA-positive cells were strongly detected in spermatogonia and in early spermatocytes in 40 (Fig. 4B and E) and 80 mg (Fig. 4C and F) dose groups of CDRI-08-treated mice to that seen in controls (Fig. 4A and D). The proliferation index was significantly increased in testicular sections of mice treated with 40 and 80 mg doses of CDRI-08 compared to controls (Fig. 4G). The number of PCNA-positive

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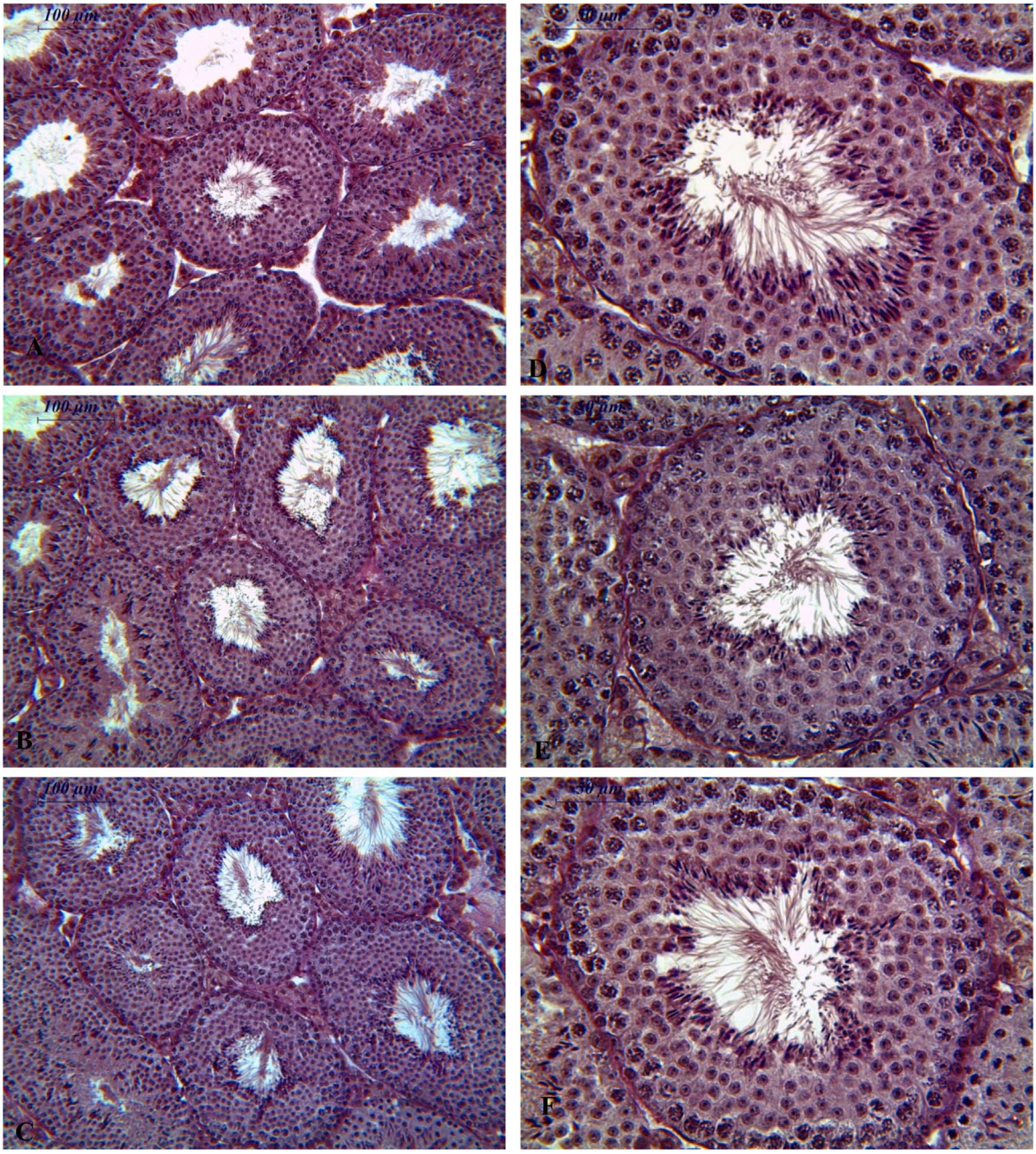

Fig. 2. PAS-haematoxylin stained sections of mouse testis (A–F). (A and D) Control showing normal appearance of the seminiferous tubules. After CDRI-08 administration, (B and E) 40 mg kg^{-1} body weight and (C and F) 80 mg kg^{-1} body weight, for 28 days. Note the normal features in the seminiferous tubules. Original magnification (A–C) × 100; (D–F) × 250.

Table 3
Diameter of seminiferous tubules, height of germinal epithelium and level of lipid peroxidation in testis, and serum level of testosterone in mice after CDRI-08 treatment.

	Group I	Group II	Group III
Tubular diameter (μm)	206.55 ± 2.62	210.41 ± 8.29	253.23 ± 12.19[a]
Height of germinal epithelium (μm)	55.03 ± 4.22	67.15 ± 2.65[a]	69.93 ± 3.76[a]
Lipid peroxidion level (nmoles TBARS/mg protein)	5.97 ± 0.83	3.40 ± 0.29[a]	3.35 ± 0.66[a]
Serum testosterone (ng/ml)	4.93 ± 0.35	5.86 ± 0.3	4.80 ± 0.23

Values are mean ± S.E.M. for five animals.

[a] Significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

cells in mice treated with 40 and 80 mg doses of CDRI-08 was 82.17 ± 1.56 and 84.05 ± 3.51 respectively, and that in controls it was 59.85 ± 2.09.

4. Discussion

The results of the present study indicate that the CDRI-08 treatment had no effect on body weight, weights of the testis, epididymis and seminal vesicle, and on histoarchitecture of the testis, though an increase in germinal epithelial height and tubular diameter was noted in the testis of the treated mice compared to controls. PCNA, a well-known 36 kDa nuclear matrix protein, is widely used in the identification of proliferating spermatogonia

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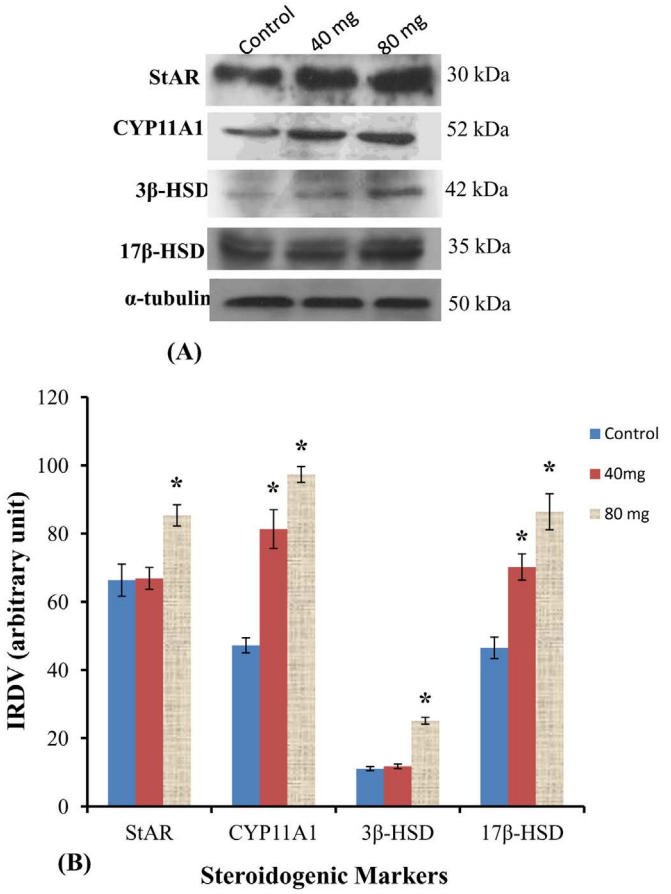

Fig. 3. (A) Western blot analyses of StAR protein and enzymes involved in testosterone biosynthesis (CYP11A1, 3β-HSD and 17β-HSD) in testes of mice after CDRI-08 administration, 40 and 80 mg kg^{-1} body weight for 28 days. (B) The densitometric data are presented as mean of integrated relative density value (IRDV) ± SEM for triplicate blots. *Significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

and spermatocytes (Steger et al., 1998; Schlatt and Weinbauer, 1994; Godlewski et al., 1999 cited in Jarvis et al., 2005). The increased number of PCNA-positive germ cells in testicular sections of CDRI-08-treated mice compared to controls as noted in P mice in the present study suggests that the treatment causes an increase in proliferating activity of germ cells, thereby a positive influence on the spermatogenic process. The immunohistochemical observation on PCNA is also supported by immunoblot study in that the expression level of this protein was significantly increased in the testis of CDRI-08-treated mice compared to controls. Thus, the increased proliferating activity of germ cells may account for an increase in the tubular diameter and the germinal epithelial height in testes of CDRI-08-treated mice in the present study.

Apoptosis, a process of programmed cell death, plays an indispensable role during spermatogenesis in that the increased numbers of germ cells undergo this process to maintain a proper balance between the germ cells and Sertoli cells to ensure that the Sertoli cell function is not compromised by excessive germ cells (Hikim, 2016). Thus, germ cell apoptosis maintains testicular homeostasis, though the increased apoptotic activity may result in defective spermatogenesis leading to infertility. In the present study, the expression level of caspase-3, one of the key executioners of apoptosis (Hikim, 2016), was not affected by the treatment; this suggests that the CDRI-08 treatment did not interfere with the normal apoptotic process.

Spermatogenesis and steroidogenesis in the testis are highly susceptible processes to the oxidative stress (Aitken and Roman, 2008). Further, oxidative stress has been identified as one of the leading factors that affects fertility status and that this results because of the imbalance between the production of reactive oxygen species and the antioxidant defense mechanisms (Makker et al., 2009). Lipids are the prime target of free radicals, implicating that cell membranes with high levels of unsaturated fatty acids are especially at risk. Thus, spermatozoa are more vulnerable to peroxidative damage because they contain high concentration of polyunsaturated fatty acids. Peroxidation of sperm lipids is reported to destroy the structure of lipid matrix in sperm membranes which causes rapid loss of intracellular ATP leading to axonemal damage, a decrease in sperm viability and an increase in morphological defects in spermatozoa (Turk et al., 2007 cited in Ceribasi et al., 2010). In P mice in the present study, CDRI-08 treatment caused a marked reduction in the level of lipid peroxidation and this observation is concomitant with the findings of sperm quality in that we noted a significant increase in viability and, on the other hand, a decrease in morphological abnormalities in spermatozoa.

It is well known that spermatogenesis is a testosterone-dependent process (O'Donell et al., 2006; Smith and Walker, 2014). StAR and CYP11A1 are believed to be the rate limiting factors in steroidogenesis, which are responsible for transport of cholesterol from outer to the inner membrane of mitochondria (Stocco, 2001), and for conversion of cholesterol to pregnenolone (Stocco and Clark, 1996), respectively. Further, the two enzymes, viz. 3β- and 17β-HSD also play key role in steroidogenic pathway in Leydig cells; 3β-HSD catalyzes the conversion of pregnenolone to progesterone and of dehydroepiandrosterone to androstenedione, while 17β-HSD converts androstenedione to testosterone. In the present study, no appreciable change was found in serum level of testosterone in CDRI-08-treated mice compared to controls. However, the same treatment did cause significant increase in testicular expressions of StAR, CYP11A1, 3β- and 17β-HSD; these observations are suggestive of positive influence of CDRI-08 on steroidogenic indices.

Plants have proved useful in the treatment of male infertility or the sexual disorders (Chauhan et al., 2014). In general, beneficial effects of the medicinal plants are ascribed to their antioxidative and/or androgenic properties (Nantia et al., 2009). For example, aqueous stem extract of *Massularia acuminata* has been shown to possess androgenic activity, which may enhance testicular functions (Yakubu et al., 2008). Likewise, treatment with *Garcinia kola* seed improves sperm characteristics in male rats accompanied by a significant decrease in lipid peroxidation level in testes and spermatozoa (Farombi et al., 2013). In P mice in the present study, CDRI-08 treatment caused an improvement in sperm quality, spermatogenic cell density and in steroidogenic parameters, and these improvements are likely to have been caused because of the reduced oxidative load in the testis, as indicated by decreased lipid peroxidation level in the gonad.

It is relevant to mention here that in our earlier publication (Singh and Singh, 2009), we have reported that treatment with Bacopa (purchased locally from the drug store under the trade name of Brahmi; Himalaya Drug, Banglore, India) caused reversible suppression of fertility in male mice; the treatment also caused reduction in motility, viability and in the number of spermatozoa accompanied by an increase in the number of morphologically abnormal spermatozoa in the cauda epididymidis of the treated mice compared to controls. In the present study, however, the results show that the CDRI-08 treatment has a positive influence on reproductive indices of male mice. It is difficult to offer an explanation for the differential effects of Bacopa, though it should be noted that the dosage regimen used in the earlier study (Singh

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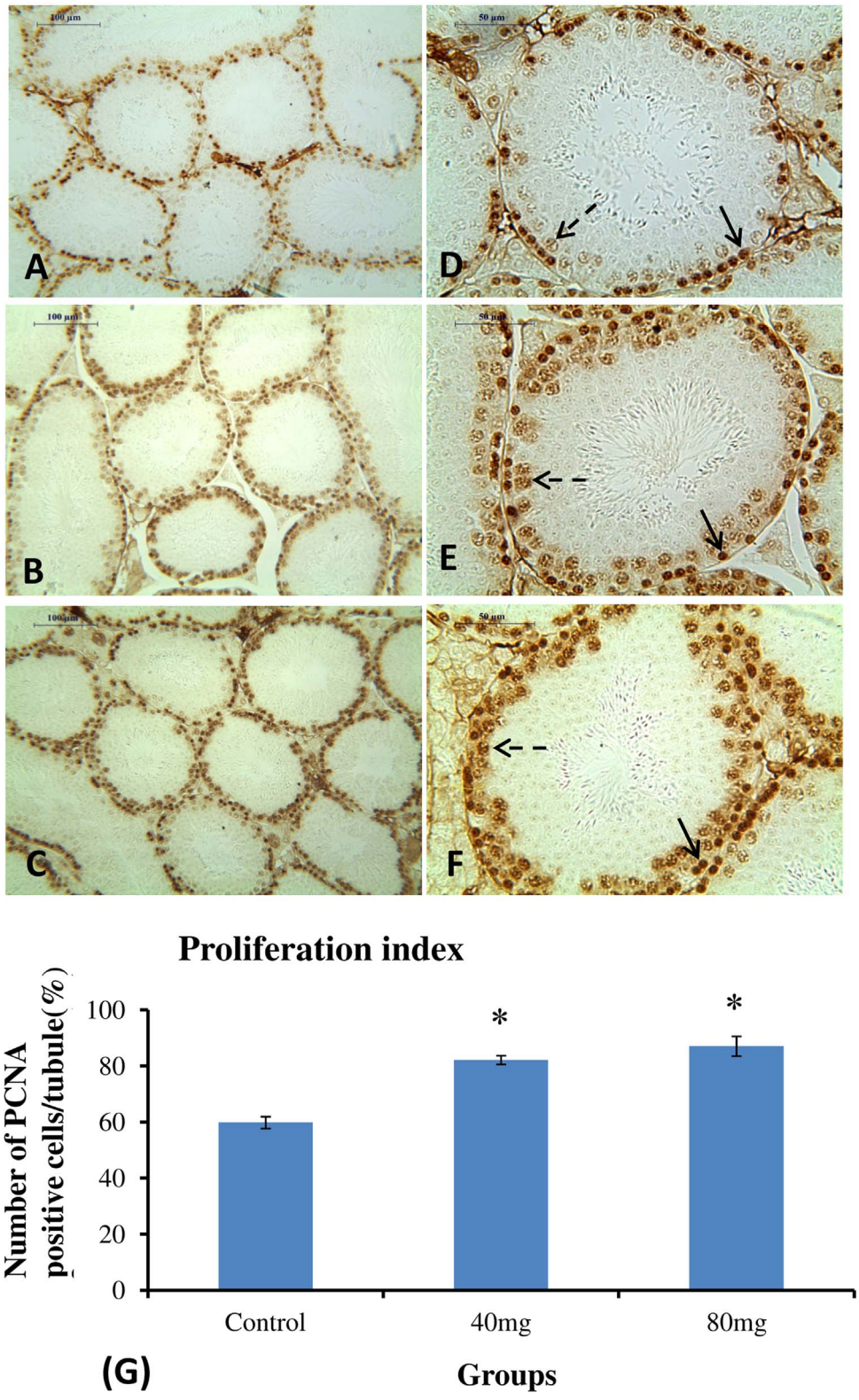

Fig. 4. Immunohistochemical analysis of PCNA in testes of control (A and D) and CDRI-08 treated groups (B and E: 40 mg kg^{-1} body weight; C and F: 80 mg kg^{-1} body weight) of mice. PCNA-positive cells are increased in 40 and 80 mg kg^{-1} body weight dose groups of CDRI-08-treated mice. Positive signals are indicated by arrows (spermatogonia) and broken arrows (spermatocytes). Original magnification (A–C) × 100; (D–F) × 250. (G) Number of proliferating germ cells per tubular cross section. Values are mean ± SEM (n=5). *Significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

and Singh, 2009) was 250 mg kg^{-1} body weight/day for 28 and 56 days, while in the present study it is 40 and 80 mg kg^{-1} body weight/day for 28 days. However, the exact reason(s) for this differential effect remains unknown and needs further studies.

5. Conclusion

In conclusion, the results of the present study suggest that treatment with CDRI-08 extract causes an improvement in sperm

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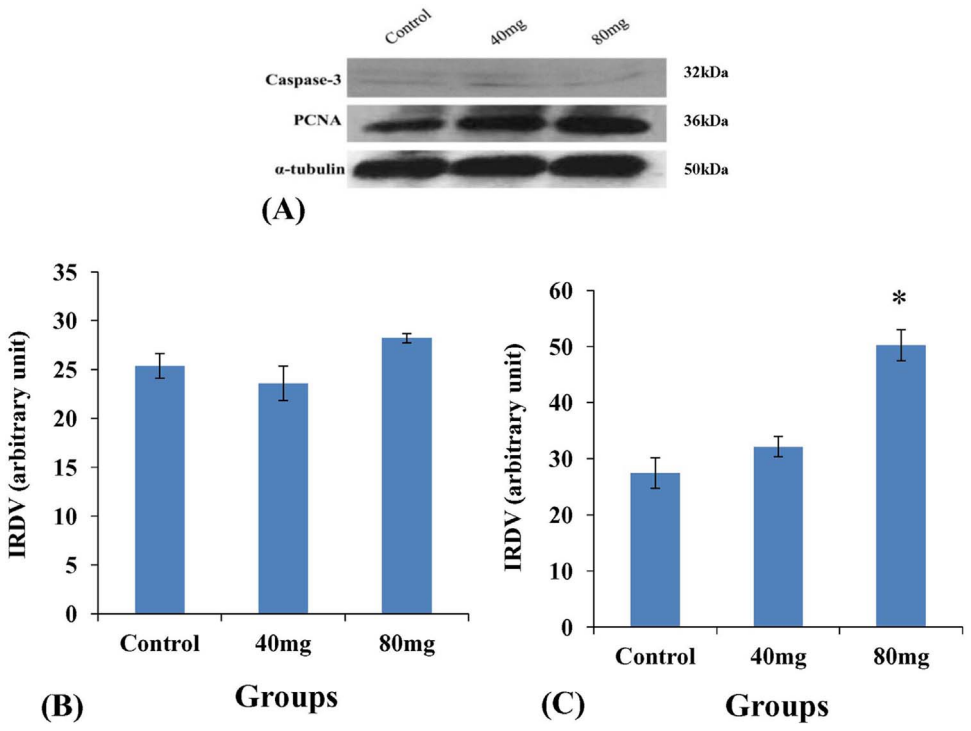

Fig. 5. (A) Western blot analyses of Caspase-3 and PCNA in testes of mice after CDRI-08 administration, 40 and 80 mg kg^{-1} body weight for 28 days. The densitometric data of Caspase-3 (B) and PCNA (C) are presented as mean of integrated relative density value (IRDV) ± SEM for triplicate blots. *Significantly different from controls ($p < 0.05$) by ANOVA followed by Newman-Keuls' multiple range test.

quality, spermatogenic cell density and in steroidogenic indices, accompanied by reduction in the peroxidative damage to the testis.

Acknowledgements

The authors are thankful to Mr. S. Selvam, CEO, Lumen Marketing Company, Chennai, India for financial support. This work was also supported by funds from the University Grants Commission through CAS in Zoology, Banaras Hindu University, Varanasi, India.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.07.026.

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