Journal of Ayurveda and Integrative Medicine 15 (2024) 100930

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: elsevier.com/locate/jaim

Shilajit mitigates chemotherapeutic drug-induced testicular toxicity: Study on testicular germ cell dynamics, steroidogenesis modulation, and Nrf-2/Keap-1 signaling

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ARTICLE INFO

Keywords:
Cyclophosphamide
Shilajit
Oxidative stress
Steroidogenesis
Testicular germ cell dynamics
Sertoli cell function

ABSTRACT

Background: Medications, including chemotherapeutic drugs, contribute to male infertility as external factors by inducing oxidative stress in testicular cells. Shilajit is a naturally occurring bioactive antioxidant used in Ayurvedic medicine to treat a variety of ailments.
Objective: This study examines the potential of Shilajit to counteract the negative effects of the chemotherapeutic drug cyclophosphamide (CPA) on testicular germ cell dynamics.
Material and methods: Male Parkes mice received single intraperitoneal CPA injection (200 mg/kg BW) on day one, followed by daily supplementation of Shilajit (100 and 200 mg/kg BW) for one spermatogenic cycle.
Results: CPA adversely affected testicular germ cell dynamics by inhibiting the conversion of spermatogonia-to-spermatids, altering testicular histoarchitecture, impairing Sertoli cell function and testicular steroidogenesis, and disturbing the testicular oxido-apoptotic balance. Shilajit supplementation restores testicular germ cell dynamics in CPA-exposed mice, as evidenced by improved histoarchitecture of the testis. Shilajit improves testicular daily production and sperm quality by promoting the conversion of spermatogonia (2C) into spermatids (1C), stimulating germ cell proliferation (PCNA), improving Sertoli cell function (*N*-Cadherin and β-Catenin), and maintaining the Bax/Bcl2 ratio. Additionally, Shilajit enhances testosterone biosynthesis by activating enzymes like 3β-HSD, and 17β-HSD. Shilajit also reduces testicular oxidative stress by increasing antioxidant enzyme activity (SOD) and decreasing lipid peroxidation (LPO). These effects are mediated by upregulation of the antioxidant protein Nrf-2 and downregulation of Keap-1.
Conclusion: The findings underscore the potent androgenic and antioxidant characteristics of Shilajit, as well as its ability to enhance fertility in cases of testicular damage caused by chemotherapeutic drugs.

1. Introduction

Sexual health is essential for overall physical and emotional well-being [1]. Over the past three decades, male infertility has emerged as a significant global health concern, affecting approximately 12–15% of couples worldwide. Notably, male factors contribute to 50% of all infertility cases [2,3]. Among the various factors responsible for the rising incidence of male infertility, the use of medications has been identified as a common external contributor to male infertility [2,4]. Chemotherapy drugs, which have played a significant role in the recent increase in cancer patient survival rates, are designed to target and kill rapidly dividing cancer cells; however, they can also affect other rapidly dividing cells in the body, such as proliferating germ cells in the testes [5,6]. Chemotherapeutic drugs cause excessive oxidative stress [7,8], which appears to have a negative impact on spermatogenesis, testicular steroidogenesis and may also impair Sertoli cell function related to the maintenance of blood-testis barriers [9,10]. This can lead to long-term or permanent male infertility, which can lead to depression, mood swings, and psychological instability in cancer survivors, preventing them from having a normal family life [11,12]. Therefore, shielding the testicular germ cell dynamics from the side effects of cancer treatments may be a promising way to preserve male fertility while undergoing cancer treatment. Recent research suggests that supplementing with antioxidants may be a viable approach to reducing the toxicity caused by

Peer review under responsibility of Transdisciplinary University, Bangalore.
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https://doi.org/10.1016/j.jaim.2024.100930
Received 5 October 2023; Received in revised form 27 February 2024; Accepted 3 April 2024

chemotherapeutic drugs [13]. Furthermore, naturally occurring antioxidant elements have been shown to be more effective than synthetic antioxidants in mitigating a variety of oxidative stress-related conditions, including male reproductive health disorders [14,15].

Shilajit is a blackish-brown herbomineral natural medicine collected from high-altitude Himalayan rocks of the Indian subcontinent [16,17]. Its chemical composition has been well characterized, and it has been discovered to contain a variety of beneficial components. These include Fulvic Acid and Humic Acid, which are said to be responsible for its health benefits. Shilajit also contains Dibenzo–Pyrones (DBPs), which have antioxidant properties. It also contains amino acids, tannins, phytosterols, and terpenoids, as well as over 40 trace minerals [18–20]. Shilajit has been shown in current scientific studies to have variety of pharmacological properties, such as antioxidant, antidiabetic, anti-aging, rejuvenating, and anti-inflammatory [18,21,22]. In addition, current scientific research also points the role of Shilajit in the prevention of cancer [23,24]. Shilajit is an important revitalising agent in the ancient Indian healing system of Ayurveda. Traditional practitioners in the Indian subcontinent have used its properties to address male sexual health issues [25]. Some scientific studies also support the beneficial effects of Shilajit on male reproductive health [18,21]. Furthermore, our earlier investigations revealed that Shilajit has the potential to safeguard male reproductive health from heavy metal exposure [25,26]. Despite being renowned for its antioxidant, rejuvenating, and anti-cancer properties, there has been a lack of comprehensive studies examining the potential of utilizing Shilajit as a natural antioxidant to mitigate the side effects of chemotherapeutic drugs on testicular germ cell dynamics.

The objective of this current study was to explore the potential of Shilajit in safeguarding testicular germ cell dynamics from the side effects of chemotherapeutic drugs. We specifically chose cyclophosphamide (CPA), a widely recognized chemotherapeutic agent with established antitumor and immunosuppressive properties [27,28]. CPA is used to treat acute and chronic leukaemias, multiple myeloma, lymphoma, rheumatoid arthritis, and ovarian and breast cancer [29,30]. However, due to its nonspecific action, it affects rapidly dividing testicular cells by inducing excessive oxidative stress, thereby leading to infertility in cancer survivors [31,32]. This underscores the need to explore the potential of a well-known natural antioxidant and rejuvenator, Shilajit, in preventing these detrimental effects. Therefore, we employed various techniques, including histology, sperm analysis, immunoblot analysis, flow cytometry, biochemical estimations, and ELISA, to evaluate the efficacy of Shilajit in maintaining testicular germ cell dynamics in male mice exposed to cyclophosphamide. The objective was to establish Shilajit as a natural remedy capable of shielding the testes from the side effects associated with anticancer medications.

2. Materials and methods

2.1. Chemicals

In the present investigation, unless otherwise indicated, chemicals were purchased from Sigma, Merck India Ltd. and SRL Pvt. Ltd, India.

2.2. Test materials and preparation of dose

Purified Shilajit (Shilajit-964; Batch No.:20201001) was obtained from VCA Healthcare Pvt Limited (G-91 Agro Food Park, MIA, Alwar, Rajasthan, India), with percentages of total fulvic acid and dibenzopyrones of 40% and 5%, respectively (according to the manufacturer's certificate of analysis). Shilajit (100 or 200 mg/kg body weight) was dissolved in double distilled water and given orally for one spermatogenic cycle (35 days). Cyclophosphamide (Endoxan; Batch No.: BUX1023) was purchased from Baxter Oncology GMBH (Westfalen, Germany). The drug was dissolved in normal saline, and a single intraperitoneal (ip) dose of cyclophosphamide at a concentration of 200 mg/kg of body weight was administered to mice on the first day of the experiment. The doses of cyclophosphamide were chosen based on Oyagbemi et al. [14]while the Shilajit doses were chosen based on our previous studies [25,26].

2.3. Animal care, maintenance and experimental design

Forty Parkes (P) strain adult male mice (14–15 weeks old) weighing 36g–42 g were used for the current study. The mice were kept in clean and hygienic conditions within a well-ventilated room, maintaining a temperature of 25 ± 2°C and a 12-h light-dark cycle. The relative humidity was maintained at 50 ± 10%. The mice were provided with pellet meal (VRK Nutritional Solutions, Laboratory Animal Feeds, Maharashtra, India) and access to water as required. Animals of each experimental group were housed in a different set of polypropylene cages (29 cm × 22 cm x 14 cm) with dry rice husk used as a bedding material. The Animal Ethics Committee (BHU/DoZ/IAEC/2020/02/035) of Banaras Hindu University approved the experimental design. Mice were randomly allocated to one of four experimental groups (10 mice/group) as follows:

1. Control: Vehicle control (200µl DDW/oral/day for 35 Days)
2. CPA: Cyclophosphamide (200 mg/kg BW, single ip injection on first day of experiment)
3. CPA + S100: Cyclophosphamide (200 mg/kg BW, single ip injection on first day of experiment) followed by Shilajit treatment (100 mg/kg BW/day) for 35 days
4. CPA + S200: Cyclophosphamide (200 mg/kg BW, single ip injection on first day of experiment) followed by Shilajit treatment (200 mg/kg BW/day) for 35 days

2.4. Sample collection

The mice were euthanized 24 h following the final administration of Shilajit. At autopsy, coronary blood samples were collected after recording the body weight of each animal. The blood samples were then processed to obtain serum which was preserved at −80 °C for later analysis. The testes were surgically excised, carefully cleaned of any residual blood precisely and weighed to an accuracy of 0.10mg. Histology and immunohistochemical localization were performed on one testis from each of five different mice. The contralateral testis of five mice were utilized to evaluate oxidative stress markers and to perform immunoblot analysis. Remaining five testes from five different mice were employed to assess daily sperm production. Contralateral testis from five different mice was used for flow cytometry analysis (see Supplementary Fig. 1). Cauda epididymidis sperm analysis was performed on six animals.

2.5. Sperm analysis (count, motility, viability and morphology)

Preparation of Sperm Suspensions: Sperm suspensions were prepared by mincing the caudal epididymis in 0.5ml of normal saline at 37 °C to assess treatment effects on sperm parameters. The suspensions (n = 6) underwent examination for motility, viability, and sperm concentration using established techniques [33–35].

Sperm Count: The sperm count was computed using the WHO laboratory manual [35]. A haemocytometer with enhanced Neubauer ruling was used. Using a WBC pipette, the sperm suspension was diluted 20-fold with normal saline. One drop of the diluted suspension was placed on each side of the haemocytometer and allowed to settle for 30 min in a humid chamber. Spermatozoa were counted in four corner squares using a microscope at 250× magnification.

Spermatozoa Concentration = N X 0.2 × 10^6 [Average number of spermatozoa counted (N) X multiplication factor (10,000) X dilution factor (20)]

Sperm Motility: Sperm motility was investigated immediately after the animals were sacrificed. A drop of sperm suspension was applied on

a clean glass slide and covered with a coverslip. The slide was kept at 37 °C for roughly a minute before being inspected with a 400× microscope. Sperm motility was assessed from various perspectives, with any degree of movement termed motile. Motility was calculated as the percentage of motile spermatozoa among the total observed.

Motility (%) = (Number of motile spermatozoa / Total number of spermatozoa) × 100

Sperm Viability: Sperm viability was evaluated using the supravital staining technique. Eosine-nigrosine stain was used, and the glassware was kept at 37 °C. A drop of sperm suspension was combined with eosin-nigrosine stain (1% eosinY+ 10% nigrosine; 1:1), and a smear was formed on a clean glass slide. The slide was inspected under a 400× microscope, with viable spermatozoa looking colorless and non-viable ones staining reddish. Viability was calculated as the percentage of viable spermatozoa among the total observed.

Viability (%) = (Number of viable spermatozoa / Total number of spermatozoa) × 100

Sperm Morphology: Sperm morphology was examined at 400× magnification by observing sperm suspension smeared onto a fresh glass slide. The criteria of Wyrobek and Bruce (1975) and Zaneveld and Polakoski (1977) [36,37]were employed for evaluation of sperm abnormalities. Approximately, two hundred spermatozoa from each sample were examined for morphological abnormalities, including tail and head abnormalities. Normal sperm morphology was also assessed.

2.6. Daily sperm production (DSP)

To evaluate the effects of Shilajit supplementation on DSP in CPA-exposed mice, elongated spermatids (step 14–16 spermatids) resistant to sonication were counted according to Meistrich and van Beek (1993). In brief, one testis was homogenized in 1ml of chilled distilled water and sonicated for 1.5 min. The sample was counted in a haemocytometer under a phase contrast microscope at 400X magnification. In mice spermatogenesis, developing spermatids spend 4.84 days in step 14–16. Therefore, to calculate daily sperm production, the number of steps 14–16 spermatids was divided by 4.84 [38,39].

2.7. Flow cytometry

To determine the effect of Shilajit supplementation on testicular germ cell dynamics in CPA-exposed mice, we quantified the various germ cell types involved in spermatogenesis using flow cytometric analysis on testicular tissue in accordance with our established methodology [38]. A flow cytometer (Beckman Coulter, CytoFLEX LX, United States) was employed to enumerate cells based on their DNA content, with approximately ten thousand fluorescent events recorded. The resulting DNA histograms were analyzed using CytExpert, Version 2.5 (Beckman Coulter) software. The data obtained from the analysis were presented as percentages of different germ cell populations (1C: round spermatid, 2C: spermatogonia, 4C: primary spermatocytes, S-Ph: S-phase germ cells) and germ cell ratios.

2.8. Histopathology of testis and epididymis

The testes and epididymis were removed and carefully cleaned before being fixed in fresh Bouin's fluid for histological examination in accordance with our established methodology. To assess the effects of CPA exposure and Shilajit treatment on the spermatogenic cycle, we selected 100 cross-sections of seminiferous tubules from five testes of five mice in each group. These were identified and categorised based on the stages of spermatogenesis, as outlined by Russell et al. [40]. In cases of severe impairment where accurate identification was not possible, the seminiferous tubules were assigned to stages I–V, VI–VIII, or IX–XII. Seminiferous tubule diameter and germinal epithelium height in Stage VII seminiferous tubules that were round or slightly oblique (n = 15) were also calculated by Motic software. Additionally, we also calculated the percentage of affected seminiferous tubules [33].

2.9. Immunohistochemical staining of proliferating germ cell nuclear antigen (PCNA)

In this study, immunohistochemistry was used to detect PCNA in testicular sections, following the protocol established by Patel et al. [34]. In brief, 6 μm thick testicular sections were deparaffinized, rehydrated, and rinsed in PBS. Antigen unmasking was achieved by heating the sections in 10mM citrate buffer with a 750W microwave before washing them three times with PBS. To counteract endogenous peroxidase activity, sections were treated with 3% H_2O_2 in methanol. Subsequently, a 2-h incubation with 5% normal goat serum was performed to prevent nonspecific antibody binding. Sections were then exposed to an anti-PCNA primary antibody (1:300 in PBS with 2% NGS) overnight at 4 °C. Subsequently, sections were treated with an HRP-conjugated goat anti-mouse IgG secondary antibody for 2 h after three PBS washes. Prior to counterstaining with haematoxylin, sections were incubated with DAB substrate (Vector Laboratories, CA, USA). The sections were dehydrated before being mounted with DPX. This experiment also included negative control slides, which contained 2% NGS in PBS instead of the primary antibody. The semi-quantification of PCNA staining in the testes of different treatment groups was performed by the ImageJ software. The DAB stained area for PCNA in the testis was obtained by using threshold tool of ImageJ as described previously [41] and data was presented as the percentage area of PCNA staining.

2.10. Evaluation of oxidative stress

In order to investigate the impact of various treatments on oxidative stress status of testis, a testicular homogenate {10% (w/v)} in 0.01mol/L chilled PBS at pH 7.4 was subjected to centrifugation at 2600rpm at 4 °C for 10 min. To assess lipid peroxidation (LPO), half of the resulting supernatant was employed, following the methodology described by Ohkawa et al. [42].

The remaining half of the supernatant was centrifuged again at 4 °C for 30 min at 12000rpm for 30 min. The supernatant obtained from this step was used to evaluate the activity of superoxide dismutase (SOD), following the protocol outlined by Das et al. [43]. Protein quantification was carried out utilizing Lowry's technique [44].

2.11. Serum hormone assay

Serum testosterone (catalogue number: DKO002) and estradiol (catalogue number: DKO003) levels were determined by ELISA kit (DiaMetra, Italy) following the instructions provided by the manufacturer.

2.12. Immunoblot analysis

To examine the expression of various markers in the testis, including germ cell proliferating markers (PCNA), oxidative stress markers (Keap-1 and Nrf-2), testicular steroidogenesis markers (StAR, CYP11A1, 3β-HSD, 17β-HSD, and CYP-19), apoptotic markers (Bax, Bcl-2, and Caspase-3), and Sertoli cell functional proteins (*N*-cadherin and β-catenin), immunoblot analysis was performed. Protein quantification and immunoblot analysis were conducted following the established laboratory protocol [45]. The signals were detected using the ChemiDoc system (Analytik Jena). The densitometry of immunoreactive bands was analyzed using AlphaEaseFC software (AlphaImager 2200), with β-actin serving as the loading control. The details of antibodies used in the present study and their dilution are given in Supplementary Table 1.

2.13. Statistical analyses

The data were expressed as the mean ± SEM (Standard Error of the Mean). Statistical analysis was performed using IBM SPSS Statistics 16 for Windows, employing a one-way ANOVA, followed by Duncan's multiple range test for making comparisons between different groups. Statistical significance was defined at $p < 0.05$.

3. Results

3.1. Body weight and testis weight

Body weights (both initial and final) did not differ significantly between mice exposed to CPA, those treated with Shilajit after CPA exposure, and the control group ($p > 0.05$, Fig. 1a). In terms of absolute testicular weight, CPA exposure significantly decreased it compared to the control group ($*p < 0.05$). Conversely, both doses of Shilajit treatment substantially increased the absolute testicular weight in mice exposed to CPA ($\#p < 0.05$, Fig. 1b).

3.2. Sperm analysis and testicular daily sperm production (DSP)

CPA exposure significantly reduces cauda epididymal spermatozoa motility, viability, and count while increasing morphological abnormalities ($*p < 0.05$). Abnormal tails were the most common sperm morphological abnormalities, followed by abnormal heads (Fig. 1d, Table 1). When compared to CPA-treated mice, both doses of Shilajit treatment resulted in significant improvements in motility, count, and viability, as well as decreased morphological abnormalities of cauda epididymal spermatozoa. ($\#p < 0.05$, Fig. 1c and d and Table 1).

In comparison to the control group, mice exposed to CPA had a notable reduction in testicular daily sperm production ($*p < 0.05$). Nonetheless, both low and high doses of Shilajit administration led to a substantial increase in testicular daily sperm production among mice exposed to CPA ($\#p < 0.05$, Fig. 1e).

3.3. Flow cytometry of testicular germ cells

The testes of control animals showed three distinct peaks in germ cell populations when the DNA content of various germ cells was examined: spermatogonia (2C), primary spermatocytes (4C), and total round spermatids (1C). The phase between the 4C and 2C peaks, during which cells are actively engaged in DNA synthesis, is commonly referred to as the S-Phase. CPA exposure significantly decreased the number of 1C and 4C populations as well as 1C:2C and 4C:2C germ cell ratios when compared to controls ($*p < 0.05$), however, it had no effect on the number of 2C and S-Phase cells ($p > 0.05$, Fig. 2a–c). Shilajit treatment, on the other hand, significantly increased 1C and 4C populations along with an increase in 1C:2C, 1C:4C, 4C:2C and 4C:S-Ph germ cell ratios in CPA-exposed mice ($\#p < 0.05$, Fig. 2a–c).

3.4. Histopathology of testis

The histological examination of the testis in the control mice revealed the presence of a typical and healthy histoarchitecture within the seminiferous tubules (Fig. 3a and 3e-g). However, CPA-exposed mice exhibited significant histopathological changes, including reduced germ cell count, tubule vacuolization, seminiferous epithelium loosening, and germ cell exfoliation into the tubule lumen, mainly in spermatogenic cycle stages I–VIII (Fig. 3b and 3h-j). Treating CPA-exposed mice with low-dose Shilajit improved tubular histoarchitecture by increasing germ cell count (Fig. 3c and 3k-m). Further, when CPA-exposed mice were treated with a high dose of Shilajit, the histoarchitecture of the seminiferous tubules was almost similar to that of the controls (Fig. 3d and 3n-p). Furthermore, quantitative histopathology revealed that both doses of Shilajit treatment significantly increased germinal epithelium height (GEH), seminiferous tubule diameter (STD), and decreased the affected seminiferous tubules compared to CPA-exposed mice ($\#p < 0.05$, Table 2).

3.5. Histopathology of epididymis

In all segments (I–V) of the epididymis, the histological examination of the control mice revealed normal histology (Fig. 4a–e). However, the epididymis of CPA-exposed mice displayed detectable histological changes, including vacuole-like spaces in segments II and IV of the epididymal epithelium and significantly fewer spermatozoa in the lumen of segment V (Fig. 4f–j). Shilajit treatment, in contrast, resulted in normal histoarchitecture in all segments (I–V) and a dose-dependent increase in the number of sperm cells in the lumen of segment V of the epididymis in CPA-exposed mice (Fig. 4k-o; 4p-t).

3.6. Immunohistochemical staining and Western blot analysis of proliferating germ cell nuclear antigen (PCNA)

PCNA immunolocalization in the testis of control mice (Fig. 5a) showed that this protein was expressed in spermatogonia and early spermatocytes. When CPA-exposed mice (Fig. 5b) were compared to controls, there was a significant decrease in PCNA immunostaining ($*p < 0.05$, Fig. 5f). Low and high doses of Shilajit treatment (Fig. 5c–d respectively), on the other hand, showed a significant increase in PCNA immunostaining in dose-dependent manner in the testis of CPA-exposed mice ($\#p < 0.05$, Fig. 5f). Furthermore, PCNA immunolocalization was also supplemented with Western blot analysis. Western blot analysis of PCNA revealed a significant decrease in PCNA expression in CPA-exposed mice compared to controls ($*p < 0.05$). By contrast, treatment with both doses of Shilajit significantly increased PCNA expression in CPA-exposed mice ($\#p < 0.05$, Fig. 5g–h).

3.7. Lipid peroxidation and activity of superoxide dismutase

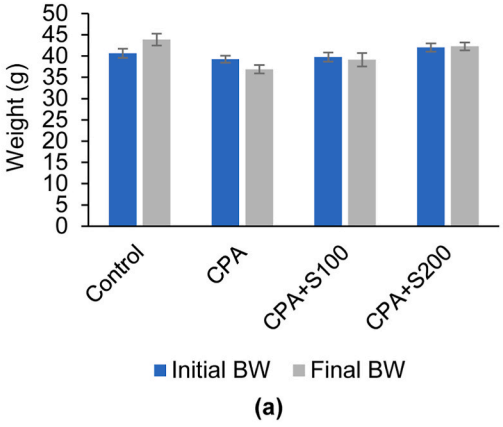
In comparison to controls, mice exposed to CPA showed a significant increase in lipid peroxidation (LPO) levels in testicular tissue ($*p < 0.05$, Fig. 5i), while superoxide dismutase (SOD) activity was significantly decreased ($*p < 0.05$, Fig. 5j). Shilajit treatment reduced LPO levels induced by CPA non-significantly ($p > 0.05$) in the testis while significantly increasing SOD levels compared to CPA-exposed mice ($\#p < 0.05$, Fig. 5i–j).

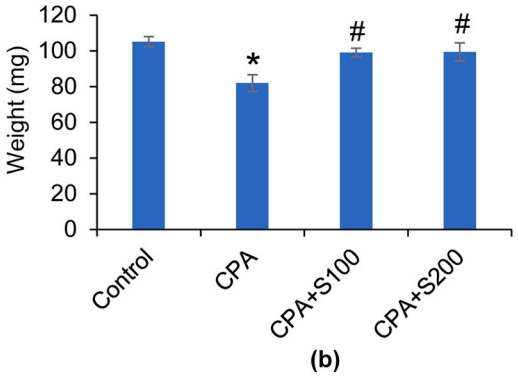
3.8. Western blot analysis of Keap-1 and Nrf-2

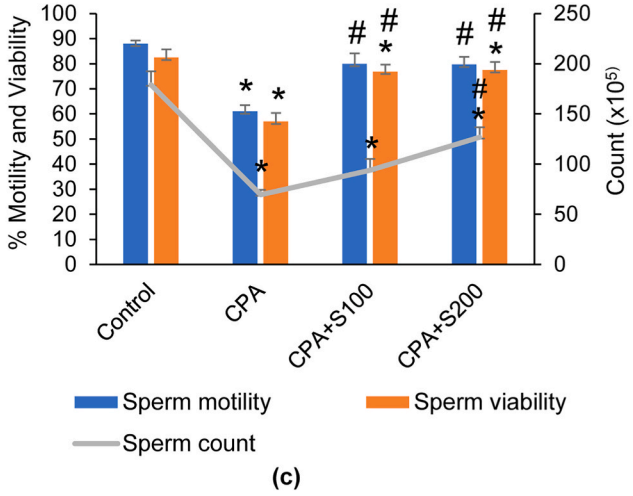
In CPA-exposed mice, the expression of Keap-1 was notably elevated compared to the control group ($*p < 0.05$). However, when treated with both doses of Shilajit, the expression of Keap-1 significantly decreased in mice exposed to CPA ($\#p < 0.05$). Conversely, Nrf-2 expression was notably decreased in CPA-exposed mice when compared to the control group ($*p < 0.05$). Nevertheless, treatment with both doses of Shilajit resulted in a significant increase in Nrf-2 expression in mice that had been exposed to CPA ($\#p < 0.05$, Fig. 5j–k).

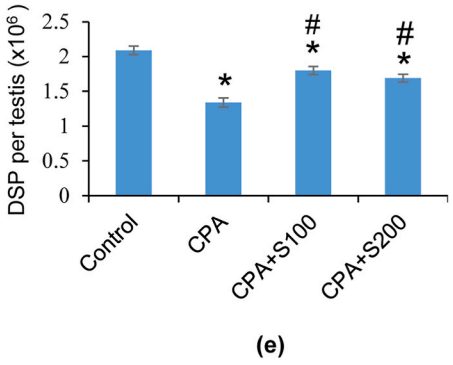
3.9. Serum testosterone and estradiol level

The serum testosterone levels of the CPA-exposed mice were significantly lower than those of the control group ($*p < 0.05$). However, treatment with Shilajit demonstrated a dose-dependent effect, leading to an increase in serum testosterone levels in the CPA-exposed mice, and the increase was significant at higher dose of Shilajit ($\#p < 0.05$, Fig. 6a). Serum estradiol levels, on the other hand, did not differ significantly between control and CPA-exposed mice ($p > 0.05$). However, Shilajit treatment decreased serum estradiol levels in CPA-exposed mice in a dose-dependent manner, and this decrease was significant in animals given a high dose of Shilajit ($\#p < 0.05$, Fig. 6b).









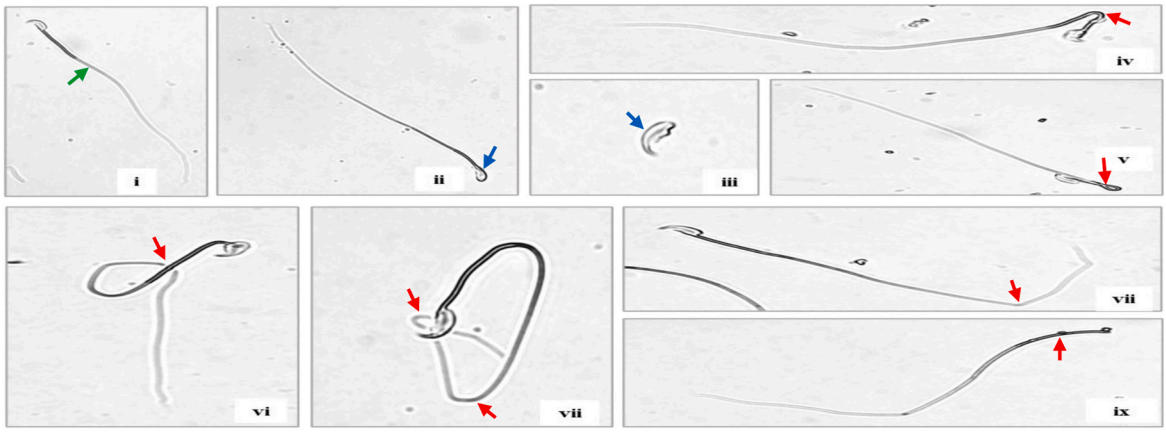


Fig. 1. Effect of Shilajit on cyclophosphamide (CPA)-exposed mice; body weight (a), absolute testis weight (b). Effect of Shilajit on sperm count, motility and viability of cauda epididymal spermatozoa of CPA-exposed mice (c). Effect of Shilajit on morphology of cauda epididymal spermatozoa of CPA-exposed mice (d). Note a normal appearance of spermatozoa (green arrow) in control mice (i) and spermatozoa from mice exposed to CPA (ii-ix). (ii & iii) head abnormalities (blue arrow): (ii) spermatozoa with amorphous head; (iii) spermatozoa with isolated head. (iv-ix) tail abnormalities (red arrow): (iv and v) spermatozoa with folded and coiled tail in proximal region, respectively; (vi) spermatozoa with coiled tail in medial region; (vii and viii) spermatozoa with coiled and folded tail in distal region respectively; (ix) spermatozoa with headless tail. Effect of Shilajit on testicular daily sperm production of CPA-exposed mice (e). * and # indicated significant different from controls ($p < 0.05$) and CPA-exposed mice ($p < 0.05$) respectively. BW: Body weight.

Table 1
Effect of Shilajit on morphology of cauda epididymal spermatozoa of CPA-exposed mice

Groups	Sperm morphology		
	Normal	Head abnormalities	Tail abnormalities
Control	88.6 ± 1.032	4.6 ± 0.925	6.8 ± 1.06
CPA	42.4 ± 0.929*	21.4 ± 0.81*	36.2 ± 0.967*
CPA + S100	55.8 ± 1.245*#	17.8 ± 0.582*#	26.4 ± 0.509*#
CPA + S200	58.2 ± 1.16*#	14.6 ± 0.747*#	27.2 ± 0.858*#

Values are mean ± SEM for 6 mice.
* and # indicated significant different from controls ($p < 0.05$) and CPA-exposed mice ($p < 0.05$) respectively.

3.10. Western blot analysis of testicular steroidogenic markers

The results of the Western blot analysis indicated consistent StAR expression across all experimental groups. However, in CPA-exposed mice treated with Shilajit, CYP11A1 expression increased while CYP19 expression decreased (#$p < 0.05$). Furthermore, CPA-exposed mice that were given a high dose of Shilajit had significantly higher levels of 3β-HSD and 17β-HSD expression in their testis than CPA-exposed mice (#$p < 0.05$, Fig. 6c–d).

3.11. Western blot analysis of apoptotic markers

In CPA-exposed mice compared to controls, Bax and Caspase-3 expression were markedly upregulated, whereas Bcl-2 expression was significantly downregulated (*$p < 0.05$). In CPA-exposed mice, the administration of Shilajit resulted in the downregulation of Bax and Caspase-3 expression, while concurrently upregulating the expression of Bcl-2 (#$p < 0.05$). Furthermore, CPA exposure significantly increased the Bax: Bcl-2 ratio (*$p < 0.05$), whereas, both doses of Shilajit treatment to these mice significantly decreased the Bax: Bcl-2 ratio in a dose-dependent manner (#$p < 0.05$, Fig. 7a–b).

3.12. Western blot analysis of Sertoli cell functional proteins

Western blot analysis revealed that *N*-cadherin expression in CPA-exposed mice was significantly lower than in controls (*$p < 0.05$), whereas β-catenin expression in these mice was comparable to controls ($p > 0.05$). However, Shilajit treatment increases the expression of both *N*-cadherin (#$p < 0.05$) and β-catenin ($p > 0.05$) in CPA-exposed mice. (Fig. 7c–e).

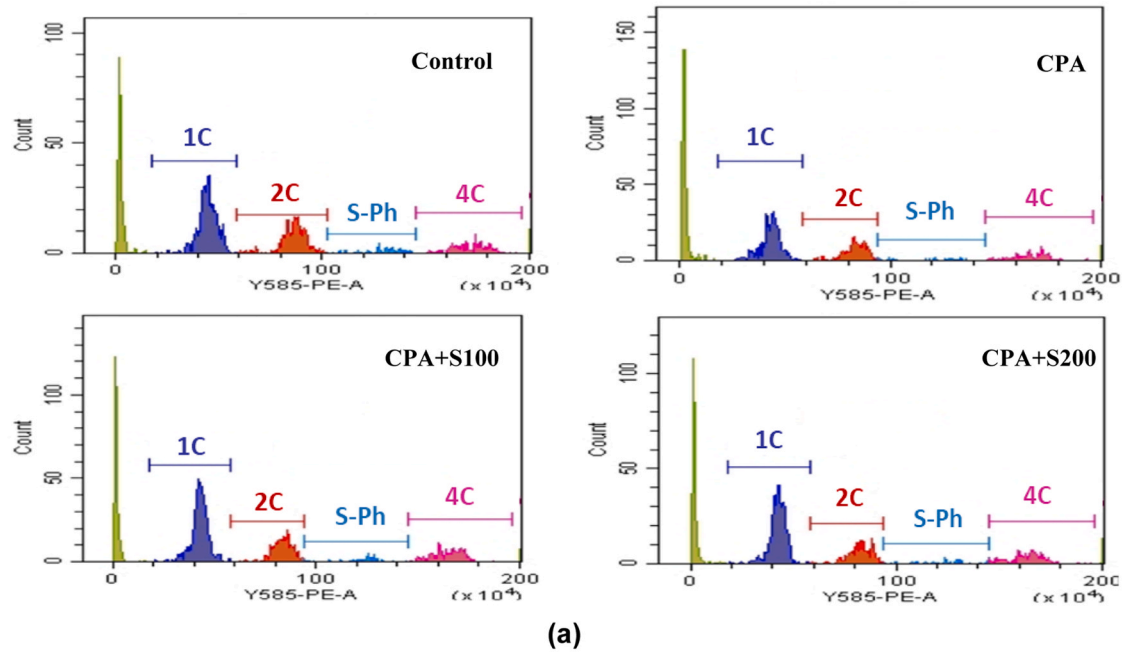
4. Discussion

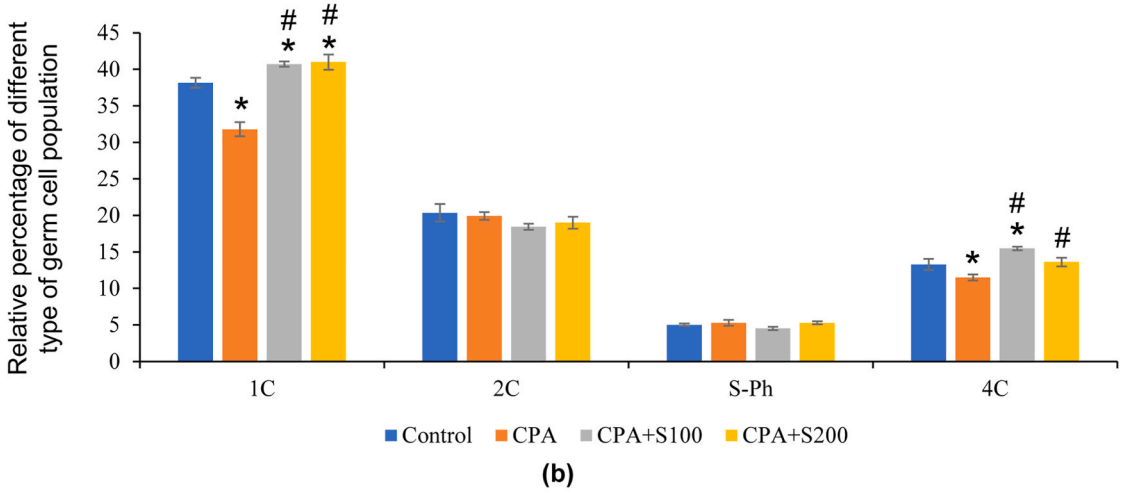
Cyclophosphamide (CPA), a commonly used chemotherapeutic drug, induces excessive oxidative stress that damages testicular cells, leading to male infertility that affects the quality of life of cancer survivors [6,30]. Therefore, there is a dire need for a safe and effective natural supplement that protects the testis from its side effects. Thus, the purpose of this study was to see whether Shilajit could protect testis from the side effects of cyclophosphamide, a well-known chemotherapeutic drug.

In Parkes mice, the acute administration of CPA did not result in notable changes in body weight across any of the treatment groups, however, testicular weight was negatively affected in mice exposed to CPA, indicating that CPA has a targeted effect on the male reproductive system [46]. While analyzing reproductive toxicity, the weight of the reproductive organs provides important information about the environment in which the reproductive organs function [47,48]. The increase in testis weight following Shilajit supplementation after CPA exposure suggests that Shilajit supplementation aids in the restoration of the functional environment of the testis, which has been harmed by CPA exposure.

CPA exposure significantly decreased serum testosterone levels in the current study by inhibiting the activities of Δ5 3β-HSD and 17β-HSD enzymes and increasing the activity of CYP19 enzymes. These findings suggest that the decrease in testosterone levels in CPA-exposed mice is caused by the negative impact of CPA on signaling pathways of testosterone biosynthesis via decreasing the secretion of LH from the pituitary gland [49–51]. In contrast, Shilajit supplementation to CPA-exposed mice reverted the repressing effects of CPA on steroidogenesis. This indicated that Shilajit might have stimulatory effects on HPG-axis by regulating the level of pituitary gonadotropins [26,52]. We found that Shilajit stimulates conversion of cholesterol to pregnanolone by stimulating the CYP11A1 enzyme and pregnenolone to testosterone by increasing the activity of Δ5 3β-HSD and 17β-HSD. Aside from this, Shilajit supplementation slows the conversion of testosterone to estradiol by decreasing the activity of the aromatase enzyme. The findings suggest that Shilajit has the ability to restore testicular steroidogenesis and serum testosterone levels in mice exposed to CPA. This is accomplished by increasing vital steroidogenic enzyme activity while decreasing aromatase activity. Furthermore, higher Shilajit dose appear to be particularly effective in mitigating the negative effects of CPA exposure on testosterone production.

In the present study, acute CPA exposure had a negative impact on the histoarchitecture of the testis. The germinal epithelium height and stage VII seminiferous tubules diameter were adversely affected. Intra-epithelial vacuolization, exfoliation of germ cells, and loosening of the germinal epithelium were frequently observed in I to VIII stages of seminiferous tubules in CPA-exposed mice. It's worth noting that major spermatogenesis activities take place in stages I-VIII, and Sertoli cells facilitate these key spermatogenic activities by interacting directly with germ cells and controlling the environmental milieu within the seminiferous tubules, and any change in Sertoli cell signaling as well as function affects the structural integrity of the seminiferous tubular epithelium [53,54]. CPA exposure adversely affected the Sertoli cell functioning by affecting the *N*-cadherin and β-catenin expression. Therefore, the loosening between the germ cell layers and exfoliation of germ cells after CPA exposure in the present experiment might be attributed to Sertoli cell dysfunction associated with compromised germ cell support and maintenance of the blood-testis barrier. The severely affected structure of seminiferous epithelium and functioning of Sertoli cells after CPA exposure as mentioned above, hampers the process of spermiogenesis and spermiation and overall spermatogenesis supported by our flow cytometric findings for the first time [55–57]. CPA exposure severely diminished 1C and 4C populations and 1C:2C, 4C:2C, 4C:S-Ph and 1C:4C ratios in mice showing overall suppression of transformation of spermatogonia to spermatids. Furthermore, the sharp decrease in the 1C population following CPA exposure indicates that post-meiotic cells are highly sensitive to the exposure. These findings are further supported by decreased daily sperm production. PCNA, a well-known biomarker, has been linked to spermatocyte and spermatogonia proliferation [58]. It is possible to explain the decrease in 4C:2C, 4C:S-Ph ratios and testicular daily sperm production after CPA exposure by evaluating the reduced numbers of PCNA-positive germ cells and PCNA expression levels, which indicate that spermatogonia and spermatocyte proliferation have been primarily affected [31]. Shilajit supplementation improves the Sertoli cell functioning and BTB integrity by improving the expression of *N*-cadherin and β-catenin along with increases in the 1C and 4C population and 1C:2C, 4C:2C, 4C:S-Ph and 1C:4C ratios. Further, increased numbers of PCNA-positive germ cells and PCNA expression levels after Shilajit supplementation at both doses in CPA-exposed mice indicate the stimulation of spermatogonia and spermatocyte proliferation in these animals and it is possible to explain the increase in height of germinal epithelium and testicular daily sperm production after Shilajit supplementation in CPA-exposed animals [52]. Testosterone regulates spermatogenesis by regulating the rate of meiosis and the association of Sertoli cells with germ cells [11]. In the absence of normal testosterone levels, spermatogenesis is slowed and





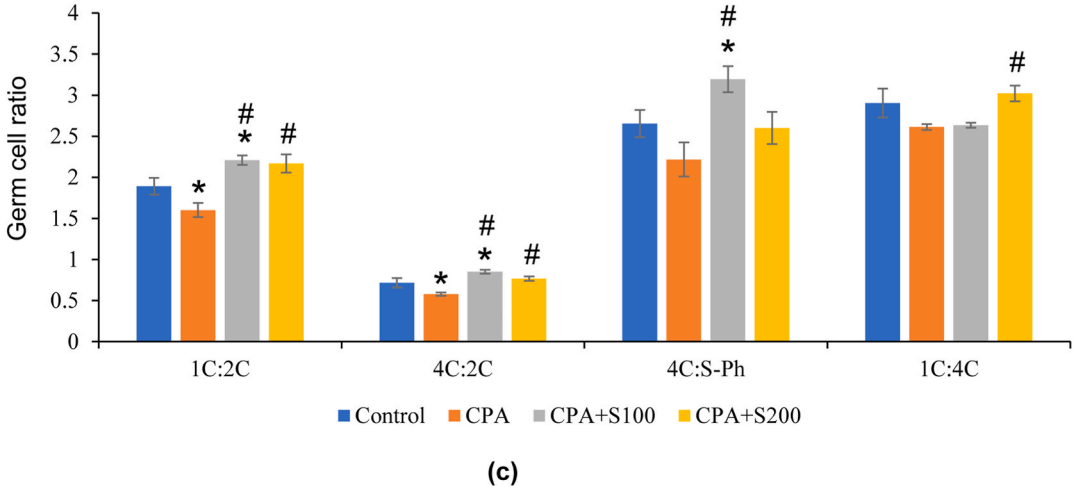


Fig. 2. A representative histogram demonstrating the distribution of DNA content in testicular germ cells in mice exposed to various treatments (a). The effect of different treatments on the distribution of different types of germ cells in testis (b) and the ratio of germ cells (c). Values are mean ± SEM. S-Ph: S-Phase. *significant difference from controls, # significant difference from CPA-exposed mice, ($p < 0.05$).

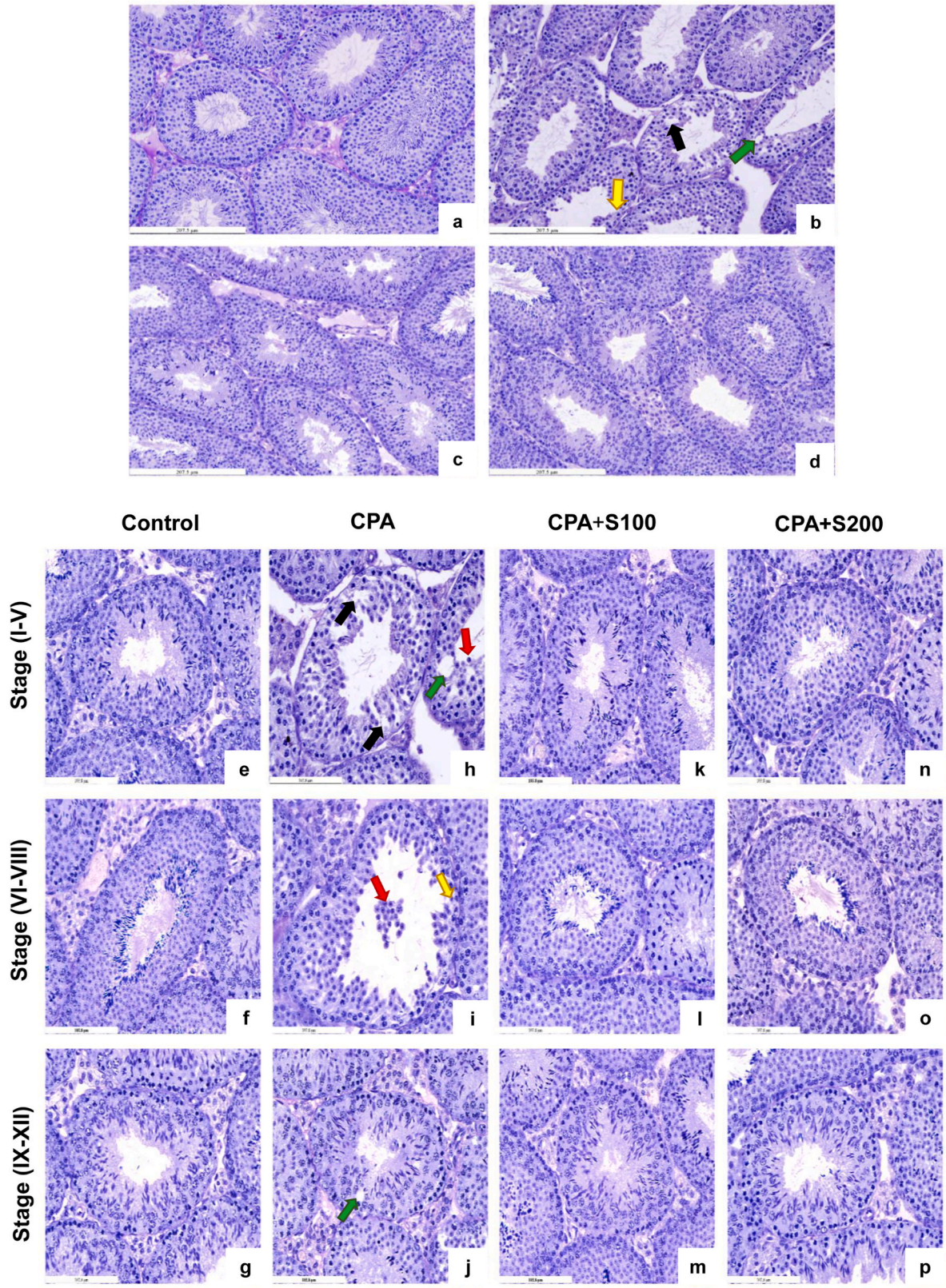


Fig. 3. Photomicrograph of seminiferous tubules of mouse testis (PAS-haematoxylin staining) (a-d; stage-specific photomicrograph e-p). Photomicrograph of testis of control mice showing normal morphological features of seminiferous tubules (a and e-g). Photomicrograph of testis of CPA exposed mice shows atrophic appearances of seminiferous tubules (b and h-j). Note the appearance of reduction of germinal epithelium height (yellow arrow), vacuolization (green arrow), germinal epithelium loosening (black arrow), and exfoliated germ cells (red arrow). However, 100 mg/kg (c and k-m) and 200 mg/kg (d and n-p) Shilajit treated CPA-exposed mice testis, showing restoration of normal histoarchitecture in seminiferous tubules. 200× (Scale bar = 207.5 μm); 400× (Scale bar = 103.8 μm).

Table 2
Effect of Shilajit on seminiferous tubules diameter (STD), germinal epithelium height (GEH) and frequency of affected seminiferous tubules of CPA-exposed mice

Groups	STD (μm)	GEH (μm)	Affected ST (%)
Control	298.2 ± 7.69	87.70 ± 1.82	11.67 ± 1.6
CPA	258.45 ± 5.56*	68.3 ± 2.02*	56.60 ± 4.4*
CPA + S100	294.43 ± 4.32#	85.43 ± 1.71#	35 ± 2.89*#
CPA + S200	292.23 ± 5.17#	85.02 ± 1.70#	31.6 ± 4.4*#

Values are mean ± SEM for 5 mice.
* and # indicated significant different from controls ($p < 0.05$) and CPA-exposed mice ($p < 0.05$) respectively.

Sertoli cell-germ cell association is disrupted [59]. By inducing steroidogenesis and inhibiting aromatase activity, Shilajit supplementation ensures that enough testosterone is available for normal spermatogenesis in CPA-exposed mice. Shilajit exerts all the above-mentioned effects by acting on local environment of the testis. According to a previous study, its active constituents dibenzo-α pyrones and fulvic acid can penetrate blood-testis barriers reach the target organs such as seminiferous tubules and exert their beneficial effects [52].

The harmony of germ cell proliferation, differentiation, and programmed cell death is essential for mammalian spermatogenesis [60]. The process of programmed cell death known as apoptosis, which takes place to preserve testicular homeostasis, is a characteristic of spermatogenic lineages [60,61]. The Bcl-2 subfamily of programmed cell death modulators promotes cell survival, whereas the Bax subfamily promotes programmed cell death [62]. A significant factor in determining cell fate has been identified as the ratio of Bax to Bcl-2. According to reports, germ cell apoptosis rates are increased by either acute or chronic exposure to CPA via increasing sperm DNA damage [62,63]. In the current study, CPA exposure also caused a disruption in the spermatogenic process by activating the pro-apoptotic factors Bax and caspase 3. The administration of Shilajit, on the other hand, demonstrated a notable reversal of this pattern. This was demonstrated by increased Bcl-2 expression and decreased Bax expression. Therefore, reducing apoptosis by diminishing the Bax/Bcl-2 ratio may also be one of the mechanisms whereby Shilajit improves testicular morphology and functions.

The epididymis is instrumental in the functional maturation of spermatozoa [64]. CPA-induced sperm DNA damage and peroxidation of polyunsaturated fatty acids on spermatozoa membranes had a negative impact on the viability, motility, count, and morphology of cauda epididymal spermatozoa [48]. The decrease in sperm count appears to

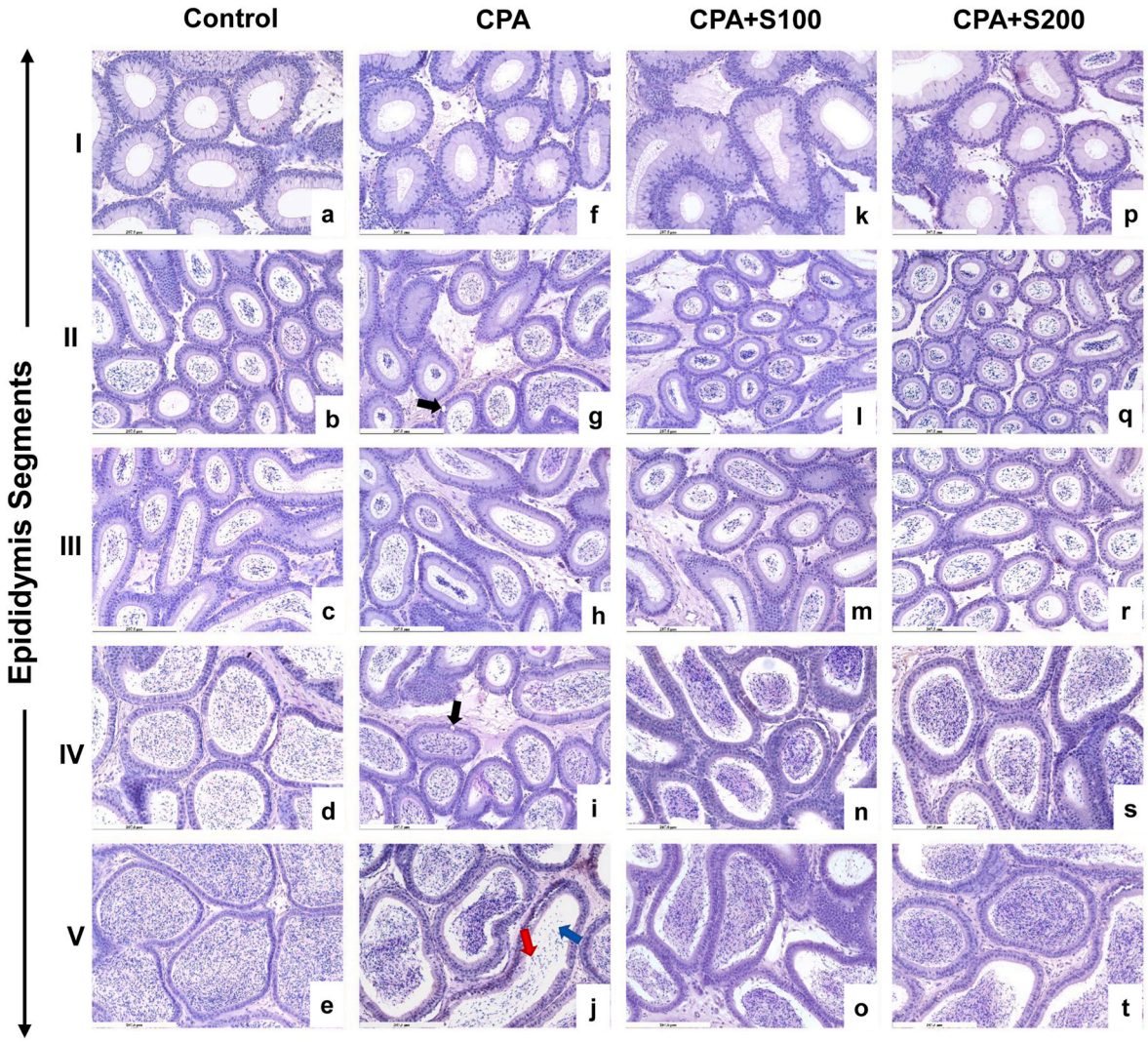
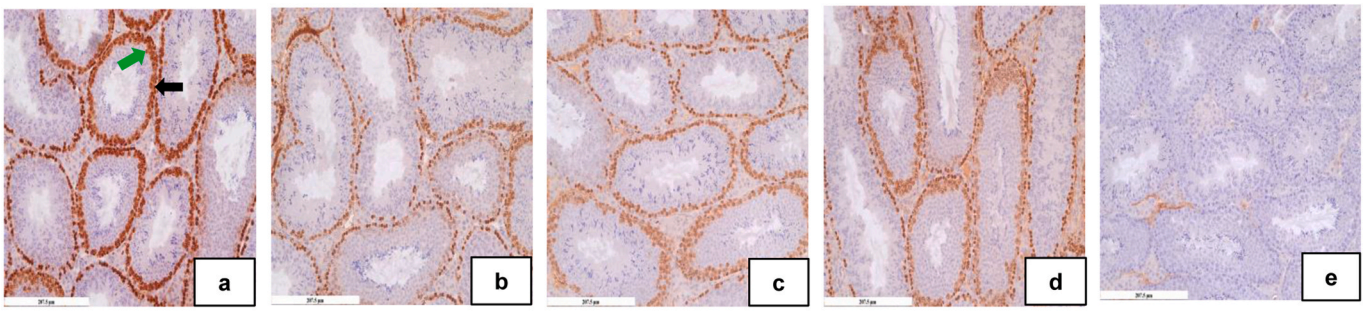
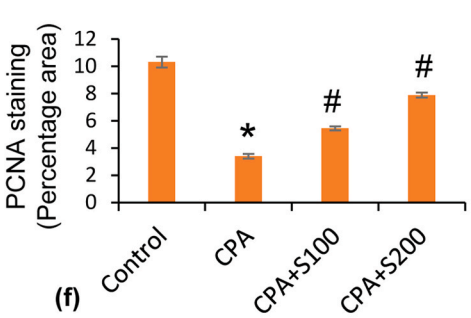
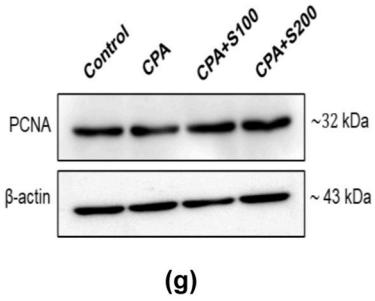
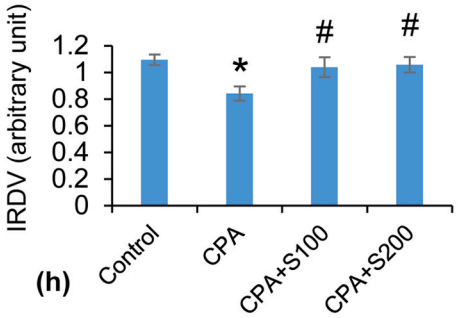


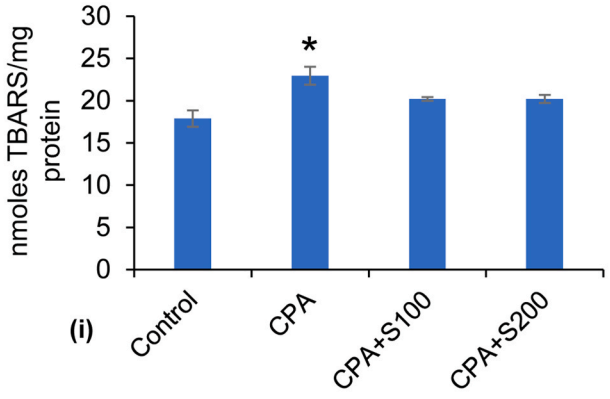
Fig. 4. Photomicrograph of mouse epididymis through segment I–V (PAS-haematoxylin staining). Control mice showing normal features (a–e). CPA-exposed mice showing vacuole-like space (black arrow) in epididymal epithelium, exfoliated germ cells (red arrow) and very less numbers of spermatozoa (blue arrow) in lumen of segment V (f–j). Shilajit treatment after CPA exposure shows a dose-dependent increase in the number of spermatozoa in lumen of segment V of the epididymis (k-o; p-t). 200× (Scale bar = 207.5 μm).

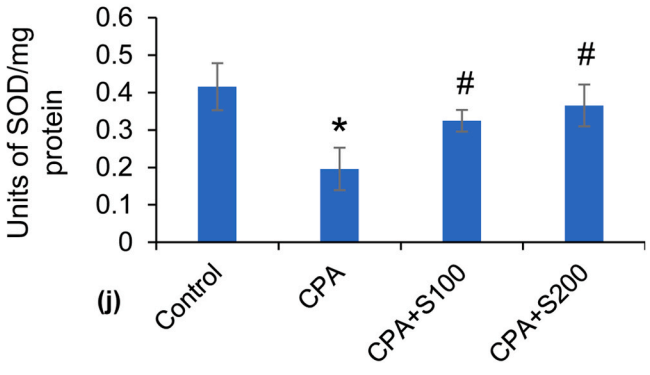


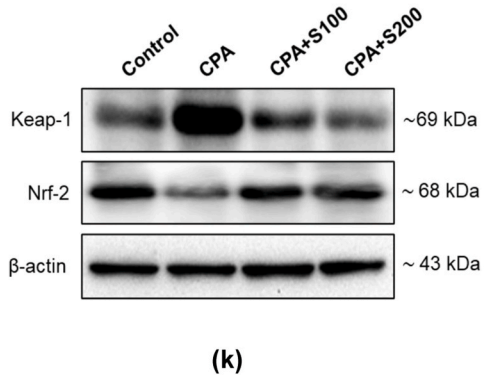












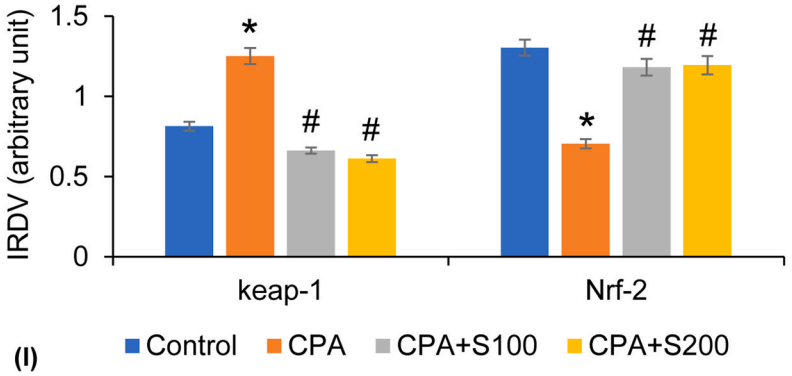


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Fig. 5. Immunolocalization of PCNA in different treatment groups. PCNA-positive cells in control mice are denoted by black and green arrows in spermatogonia and spermatocytes respectively. PCNA-immunostaining is confined nearly to spermatogonia in CPA-exposed mice (b) compared to controls (a). However, 100 and 200 mg/kg Shilajit (c and d respectively) administration results in a dose-dependent increase in PCNA-immunostaining when compared to mice that were exposed to CPA. A photomicrograph of negative control has been provided (e); 200× (Scale bar = 207.5 μm). Semi-quantification of PCNA immunostaining showed a significant decrease in CPA-exposed mice compared to controls and Shilajit significantly increased the PCNA immunostaining in the testis compared with CPA-exposed mice (f). Western blot analysis of PCNA in the testes of different treatment groups (g). The expression levels of PCNA have been normalized against its corresponding β-actin loading controls. Densitometric data of PCNA of triplicate blot are represented as mean of IRDV ± SEM (h). Effect of Shilajit on lipid peroxidation (i) and activity of Superoxide dismutase (j) of CPA exposed mice. Western blot analyses of Keap-1 and Nrf-2 in the testes of different treated groups (k). The expression levels of Keap-1 and Nrf-2 have been normalized against their corresponding β-actin loading controls. Densitometric data of Keap-1 and Nrf-2 of triplicate blot are presented as mean IRDV ± SEM (l). *significant difference from controls, # significant difference from CPA-exposed mice, ($p < 0.05$). TBARS: thiobarbituric acid-reactive substances; Keap-1: Kelch Like ECH Associated Protein 1; Nrf-2: NF-E2–related factor 2.

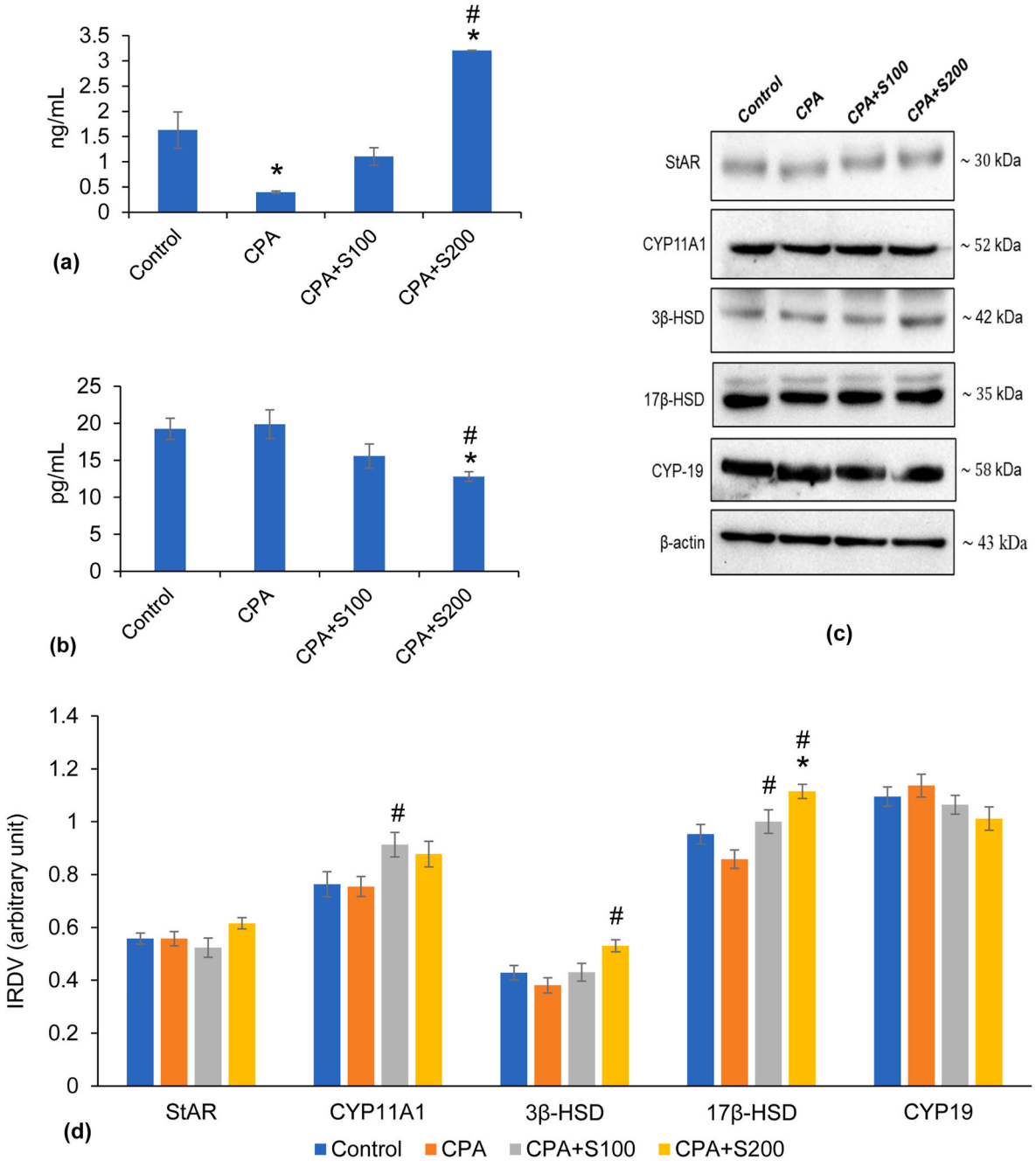

Fig. 6. Effect of Shilajit on serum testosterone (a) and serum estradiol level (b) of CPA-exposed mice. Western blot analyses of StAR, CYP11A1, 3β-HSD, 17β-HSD & CYP-19 in the testes of different treatment groups (c). The expression levels of each protein have been normalized against its corresponding β-actin loading control. Densitometric data from a triplicate blot of StAR, CYP11A1, 3β-HSD, 17β-HSD & and CYP-19 are presented as mean of IRDV ± SEM (d). * significant difference from controls, # significant difference from CPA-exposed mice, ($p < 0.05$). StAR: steroidogenic acute regulatory (protein); HSD: hydroxysteroid dehydrogenases.

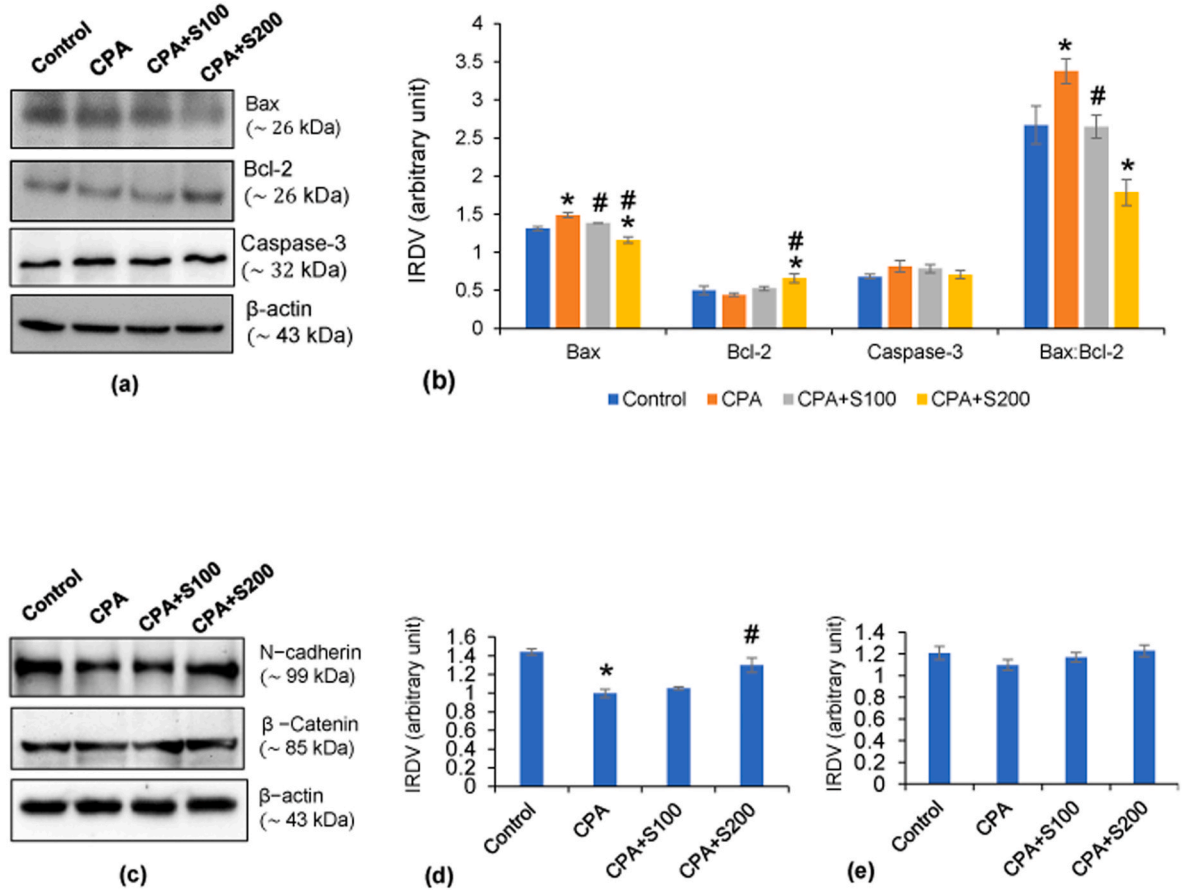

Fig. 7. Effect of Shilajit on apoptotic markers of different treated groups (a and b). Western blot analyses of Bax, Bcl-2, and caspase-3 in the testis of different treated groups (a). The expression levels of each protein have been normalized against its corresponding β-actin loading control. Densitometric data from a triplicate blot of Bax, Bcl-2, and caspase-3 are presented as the mean of IRDV ± SEM (b). Effect of Shilajit on Sertoli cell markers of different treated groups (c–e). Western blot analyses of *N*-cadherin and β-catenin in the testes of different treated groups (c). The expression levels of each protein have been normalized against its corresponding β-actin loading control. Densitometric data from a triplicate blot of *N*-cadherin and β-catenin are presented as mean of IRDV ± SEM (d and e respectively). *significant difference from controls, # significant difference from CPA-exposed mice, ($p < 0.05$).

be the result of the adverse effect of CPA exposure on the testis [47], as we observed mainly exfoliated germ cells and a few spermatozoa in the lumen of segment V of the epididymis [14,65]. Spermatozoa transported from the testes to the epididymis are functionally premature and lack motility [66,67]. Therefore, the decrease in motility and viability of spermatozoa appears to be the result of CPA on the functional environment of the epididymis, as CPA has been reported to adversely affect the functional environment of the epididymis by inducing oxidative stress [30]. CPA is known to increase lipid peroxidation in sperm [14], which is responsible for head and tail abnormalities in sperm, as evident in the current study. The adverse effects of CPA on cauda epididymal sperm motility, viability, count, and morphology were restored by Shilajit supplementation. Shilajit has a positive effect on spermatogenesis, as evidenced by the fact that lumen of segment V is filled with spermatozoa in Shilajit-treated animals, which accounts for the increase in cauda epididymal sperm count. Shilajit improves the functional environment of the epididymis by maintaining the optimum concentration of sialic acid [26,68], which may account for the dose-dependent increase in viability, motility, and a number of morphologically normal spermatozoa by Shilajit supplementation in CPA-exposed animals.

The main consequences of the use of chemotherapeutic drugs have been the disturbed redox balance of tissues by generating excessive oxidative stress in the form of reactive oxygen species (ROS) [62]. Testis is susceptible to damage from oxidative stress, so the antioxidant defence system (Nrf-2/Keap-1 system and superoxide dismutase) offers protection by directly removing free radicals from the testes [69,70]. Under normal circumstances, Nrf-2 is bound to its inhibitor protein, Keap-1, in the cytoplasm. However, during oxidative stress, Keap-1 can become oxidized or undergo covalent modifications, dissociating from Nrf-2. This allows Nrf-2 to regulate lipid peroxidation (LPO) by triggering the expression of various antioxidant enzymes and stimulating the production of superoxide dismutase (SOD), which helps prevent the formation of harmful hydroxyl radicals and enhances cellular antioxidant capacity [71,72]. In the current study, exposure to chemotherapeutic drugs, specifically CPA, resulted in a decrease in Nrf-2 expression and an increase in Keap-1 expression, leading to the suppression of SOD activity. Consequently, this imbalance disrupted the antioxidant defence system and caused oxidative stress, as indicated by an elevated level of lipid peroxidation. Shilajit is a well-known natural antioxidant that has been used in both in vitro and in vivo studies [18,21]. Shilajit at both doses improves the oxidative status of the testis in mice exposed to CPA in the current study. Shilajit increases the expression of Nrf-2, suppresses the expression of Keap-1, and increases the activity of SOD, resulting in a decrease in lipid peroxidation levels. These findings demonstrated that Shilajit had a protective effect against CPA-induced testicular oxidative damage in mice, and that its mechanism may be connected to the activation of the Nrf-2-Keap-1 signaling pathway.

5. Conclusion

In conclusion, CPA exposure exacerbates oxidative imbalance in the testis, resulting in adverse effects on testicular steroidogenesis, Sertoli

cell function, germ cell proliferation, and spermiogenesis. This adversely affected overall germ cell dynamics in the testis, as evidenced by an increase in the frequency of affected seminiferous tubules, and adversely affected sperm parameters (motility, viability, count, and morphology) and testicular daily sperm production. In contrast, our study highlights the potent antioxidant properties of Shilajit, which effectively mitigates CPA-induced testicular dysfunction by modulating the Nrf-2/Keap-1 system and restoring oxidative balance, thereby exerting antioxidant effects. Additionally, Shilajit supplementation promotes the expression of crucial steroidogenic enzymes and Sertoli cell functional proteins and facilitates the transformation of spermatogonia into spermatids. It also enhances the expression of PCNA and reduces the Bax/Bcl-2 ratio. Collectively, these effects contribute to the progression of spermatogenesis, leading to notable improvements in testicular daily sperm production and germ cell dynamics. Notably, the 200 mg/kg dose of Shilajit demonstrates significant effectiveness in enhancing steroidogenic markers, testosterone levels, anti-apoptotic protein Bcl-2, and Sertoli cell marker *N*-cadherin expression. Overall, Shilajit at both doses shows promise in mitigating CPA-induced damage in the male reproductive system, suggesting a potential strategy for managing reproductive dysfunction associated with chemotherapeutic drugs.

Sources of funding

This investigation was supported in part by the ICMR, New Delhi in the form of a major research grant (5/10/FR/29/2019-RBMCH) and by Institute of Eminence research grant (IoE, 6031) from Banaras Hindu University to RKM. AR is recipient of SRF from the CSIR, New Delhi (09/013(0899)/2019-EMR- I).

Author Contributions

Raghav Kumar Mishra: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision. **Arti Rajpoot:** Methodology, Investigation, Writing- Original draft, Data Curation, Visualization, Figures and Tables; **Kiran Yadav and Anupam Yadav:** Formal analysis, Visualization.

Declaration of Generative AI in Scientific writing

None.

Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgement

Authors are thankful to the Indian Council of Medical Research, New Delhi, for the EMR grant (5/10/FR/29/2019-RBMCH) to RKM and the Council of Scientific and Industrial Research, New Delhi, for the JRF (09/013(0899)/2019-EMR-I) to AR. We are also grateful to IoE, Banaras Hindu University, for providing financial support to RKM, and CAS, Department of Zoology, BHU, for extending their lab facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2024.100930.

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