IJPSR (2023), Volume 14, Issue 4 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 27 July 2022; received in revised form, 27 August 2022; accepted, 20 October 2022; published 01 April 2023

CYCLOPHOSPHAMIDE INDUCED DNA AND TESTICULAR DAMAGE: PROTECTIVE ROLE OF *WITHANIA SOMNIFERA*

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Keywords:

Cyclophosphamide, *Withania somnifera* extract, DNA damage

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ABSTRACT: Objectives: Cyclophosphamide is an alkylating agent known for its reproductive toxicity in males. This study evaluates the protective effects of *Withania somnifera* extract (WSE) on cyclophosphamide (CP) induced testicular toxicity and DNA damage in male rats. **Methods:** Adult male rats were randomly divided into four groups Group1-Control, Group 2-Cyclophosphamide, Group 3- *Withania somnifera* extract, Group4-Cyclophosphamide+ *Withania somnifera*. After the experimental period of 8 weeks, sperm quality, hormonal analysis, and DNA damage studies were carried out. Histopathological analysis of testes was performed for the assessment of spermatogenic disorders. **Results:** In CP-treated rats, sperm quality was reduced with decreased testosterone levels with increased DNA damage. Co-administration of WSE with CP improved sperm quality, testosterone levels, spermatogenesis and genotoxicity of cyclophosphamide. The histopathological study also restored most of the degenerative and necrotic changes. **Conclusions:** This study suggests a prominent role of WS against CP-induced testicular toxicity and DNA damage.

INTRODUCTION: With its clinical and psychosocial implications, male infertility poses a significant challenge to society. According to WHO, 13–18% of couples suffer from infertility, of which male infertility is seen in 39% of cases. Spermatogenic failure leading to azoospermia and oligospermia is one of the important causes of male infertility [1]. Cyclophosphamide is a chemotherapeutic drug known to induce testicular toxicity. Cyclophosphamide has a wide spectrum of clinical uses. It is used in treating malignancies, as an immunosuppressive, and in managing kidney diseases [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(4).1762-68

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.14(4).1762-68

Prolonged use of cyclophosphamide can lead to testicular atrophy, and side effects like oligo- or azoospermia has been reported in men [3]. Advances in chemotherapy research have been a boon to many who have cancer. The reproductive function is a serious concern in male patients within their reproductive age, who suffer from cancer, survive after tedious gruelling treatments. Chemotherapeutic agents like cyclophosphamide exert their action by inhibiting the rapidly dividing cancer cells [4].

Since, spermatogenesis is a complex process involving rapidly dividing cells, it is vulnerable to chemotherapy. Cyclophosphamide and other anticancer drugs can lead to sperm abnormalities and male infertility. The testicular damage caused by cyclophosphamide and other chemotherapeutic agents is said to be related to the dose and duration of treatment [5]. Efforts are needed to reduce these adverse effects (complications) in young patients.

Presently there is more understanding about the role of sperm DNA integrity in male factor infertility. Sperm DNA damage may have a negative effect on fertility potential. The sperm DNA damage often correlates with poor seminal parameters such as sperm count, motility and morphology [6]. Oxidative stress is associated with sperm dysfunction, a major cause of male infertility. Oxidative stress disrupts DNA fragmentation, and increased infertility rates have been found in men with seminal fluid containing high levels of reactive oxygen species (ROS) [7]. Spermatozoamembranes have an abundance of polyunsaturated fatty acid, which is highly vulnerable to oxidative stress due to lipid peroxidation. Many studies have suggested that antioxidant supplementation slightly improves sperm function and DNA integrity [8]. These ROS are associated with sperm dysfunction, germ cell DNA damage with the possibility of impaired fertility, but the exact mechanism is not completely understood.

***Withania somnifera* (Local Name: Ashwagandha, English:** Winter cherry, Indian Ginseng) has a long history stemming back to 4000 years. It is widely used in the Ayurveda, Siddha and Unani system of medicine for centuries. The medicinal uses of *Withania somnifera* are mentioned in Charaka Samhita [9]. It has rejuvenating potential, usually used as an adaptogen to combat stress, against infections and in infertility, widely used in traditional medicine. The name 'ashwagandha' means the smell of horse (Ashwa = horse, Gandha = smell) as the fresh roots smell of horse's urine, and also it imparts the sexual stamina of a horse. The species name *somnifera* originates from Latin meaning 'sleep-inducing'. They belong to Family *Solanaceae* [10]. *Withania somnifera* (Linn.) is an erect evergreen shrub that grows upto 4 feet in height, with a central stem from which branches extend, covered with dense hairs. Dry roots appear cylindrical, brownish-white exterior and appear pure white when broken. The most commonly used parts of *Withaniasomnifera* are roots and leaves [11]. Cyclophosphamide's effects on testis were evaluated in this study and its effects on DNA damage. Strategies to minimize the side effect of anticancer drugs with preserving their therapeutic efficacy are needed. In the search for new compounds, it is obvious to depend on traditional medicine. In light of these reports, the protective effects of *Withania somnifera* root extract were evaluated in CP-induced testicular toxicity.

MATERIALS AND METHODS:

Animals: Healthy adult male Wistar albino rats (200-250g), were used in this study. The Institutional Animal Ethics Committee approved the study (Ref No. KSHEMA/IAEC/13/2017). It carried it out in compliance with the guidelines laid by the Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India. Animals were housed in polypropylene cages with paddy husk bedding at room temperature. They were provided with food pellets and water *ad libitum*. The rats were marked for personal identification and were housed in cages seven days before the experimental procedure for acclimatization.

***Withania somnifera* Extract (WSE):** The *Withania somnifera* roots were procured from the local market, authenticated by a botanist, shade dried, and powdered coarsely. A weighed quantity of the powder was subjected to Soxhlet extraction with ethanol for 72 hours at a temperature of 78°C.

The resulting extract was concentrated and the solvent was completely removed by a rotary evaporator and stored in the refrigerator for future use. The percentage yield of the *Withania somnifera* extract was 20%. The extract was dissolved in distilled water and orally administered to the rats using a rat feeding tube.

Experimental Design: Animals were randomly segregated into 4 groups.

Group 1: Control received distilled water.

Group 2: Cyclophosphamide 15 mg/kg twice weekly orally.

Group 3: *Withania somnifera* extract 200mg/kg daily orally.

Group 4: Cyclophosphamide 15mg/kg twice weekly + *Withania somnifera* extract 200mg/kg daily.

Sample Collection: After 8 weeks of the experimental period, rats were sacrificed by an

overdose of ketamine 300 mg/kg i.p. Laparotomy was done and the testis was processed for histology. Whole blood collected was used for hormonal analysis and comet assay.

DNA Damage Study: COMET assay/Single cell gel electrophoresis was conducted to measure DNA damage. It detects DNA strand breaks which, when subjected to electrophoresis, will result in the migration of DNA fragments out of the nucleus to form the tail of a comet-like structure. The extent of DNA liberated from the head of the comet is the function of the dose/potency of the test agents [12].

The protocol followed was the alkaline comet assay. The whole blood 500µL was suspended in 500 µL of histopaque reagent carefully, centrifuged at 3000rpm for 10 minutes, and the middle white lymphocyte layer was collected and stored. After the preparation of microgel slides, electrophoresis was carried out for 30 min at 24 Volts and 300 mill amperes, allowing for fragmented DNA migration. After electrophoresis, the slides were neutralized with neutralization buffer, and stained with 80µL of ethidium bromide (20µg/mL). To visualize DNA damage, ethidium bromide-stained DNA was observed with a fluorescence microscope (Olympus, 40x objective).

The extent of DNA damage was assessed from the DNA migration distance and the percentage of migrated DNA. For each sample, fifty randomly selected cells were examined for each replicate. The DNA strand breaks of the stored images were quantified using Comet score software, and the parameters like the tail length, percentage of DNA in the tail, and olive tail moment (OTM) were considered to determine the level of the genotoxicity and cytotoxicity [13].

Tail length is the total length of DNA migrated from the body of the nuclear core. It is mainly used to evaluate the extent of DNA damage. Head percentage DNA is the percentage of ratio of head optical intensity (HOI) to the sum of the HOI and tail optical intensity (TOI), i.e.,

$$\text{Head\% DNA} = (\text{HOI} / (\text{HOI} + \text{TOI})) \times 100$$

Tail percentage DNA is the difference in amount of total percentage of DNA present in the comet and head percentage DNA, i.e.,

$$\text{Tail\% DNA} = 100 - \text{Head \% DNA}$$

Olive tail moment (OTM) is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail.

$$\text{Olive tail moment} = (\text{Tail. mean} - \text{Head. mean}) \times \text{tail \% DNA} / 100.$$ [14]

Hormone Analysis: The testosterone, LH, and FSH level was carried out using the ELISA kits from Xema Co Ltd.

Histopathological Analysis: Histopathological examination of the testis is acknowledged as the most sensitive method for detecting disturbances in spermatogenesis. The formalin-fixed testis was dehydrated in a series of graded ethanol, cleared in xylol, and embedded in fresh paraffin wax, followed by section cutting and staining with hematoxylin & eosin and was examined under the light microscope [15].

Statistical Analysis: Results are expressed as Mean±SD. One-way analysis of variance (ANOVA) was carried out and the statistical comparisons among the groups were performed with Tukey's multiple comparison *post hoc* test. The results with P-value <0.05 were considered significant.

The statistical analysis of the data was done using the SPPS software.

RESULTS:

Comparison of Comet Assay between Study Groups:

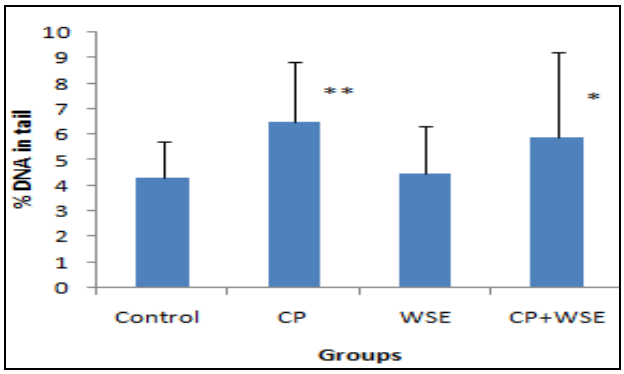

FIG. 1: % DNA IN TAIL BETWEEN STUDY GROUPS

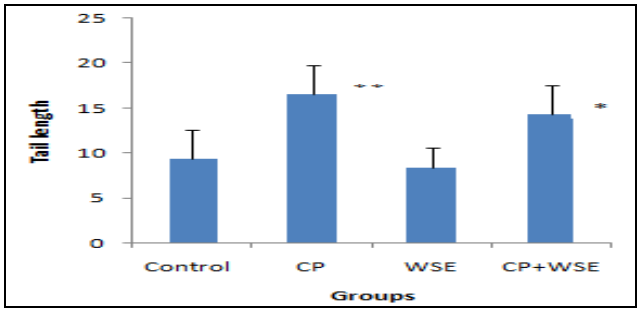

FIG. 2: TAIL LENGTH BETWEEN STUDY GROUPS

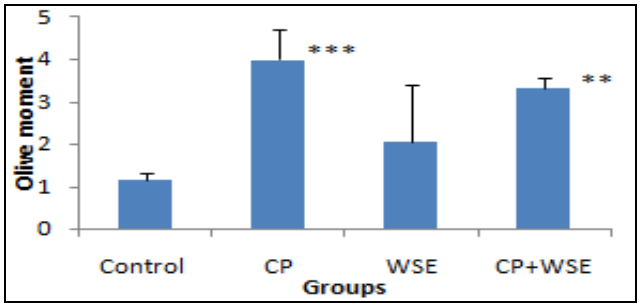

FIG. 3: OLIVE TAIL MOMENT BETWEEN STUDY GROUPS. Control: Distilled water; CP: Cyclopho-sphamide; WSE: *Withania somnifera* extract Values are expressed as Mean ± SD. One Way ANOVA followed by Tukey's multiple comparisons test. *** p<0.05 as compared with control group ** p<0.01 as compared with control group * p<0.05 as compared with control group.

TABLE 1: HORMONAL ANALYSIS BETWEEN STUDY GROUPS

Groups	Testosterone nmol/l	LH IU/l	FSH IU/l
Control	17.37± 3.15	4.32± 0.43	4.17± 0.45
CP	7.54± 1.51**	3.41± 0.34	1.01± 0.71**
WSE	18.32± 8.32	5.48± 0.83	3.07± 1.32
CP+WSE	14.36± 6.12 [a]	4.91± 2.32	3.33± 0.23*

Control: Distilled water; CP: Cyclophosphamide; WSE: *Withania somnifera* extract. Values are expressed as Mean ± SD. One Way ANOVA followed by Tukey's multiple comparisons test. ** p<0.01 as compared with control group * p<0.05 as compared with control group [a]p<0.05 as compared with CP group.

Histopathological Changes between Study Groups

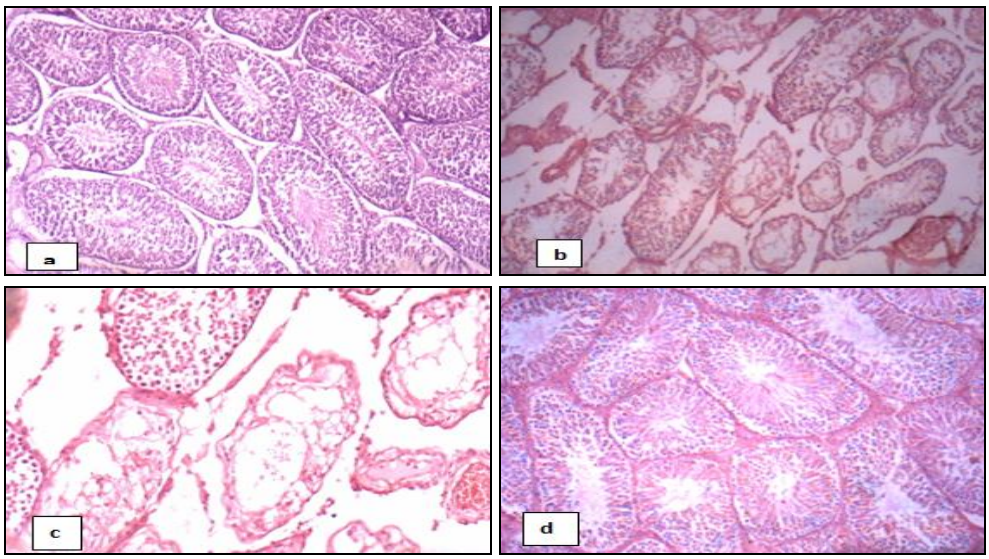

FIG. 4: REPRESENTATIVE MICROPHOTOGRAPHS OF RAT TESTIS STAINED WITH HEMATOXYLIN AND EOSIN. a- Microphotography of the control group displaying normal spermatogenesis (x 5X, H & E stain), b- CP group (x 5X, H & E stain), c- CP group displaying vacuolization & cystic degeneration of germ cells with marked reduction of germ cell count with an absence of spermatogenesis (x 10X, H & E stain), d- WS+CP displaying normal seminiferous tubules with active spermatogenesis (x 5X, H & E stain).

DISCUSSION: The long term adverse effect of chemotherapy may be more important than acute adverse effects because they affect the quality of life. Chemotherapy-induced infertility is far less frequently addressed than acute adverse effects like myelosuppression & mucositis. Male germ cells are known to be one of the tissues prone to Cyclophosphamide toxicity [16].

Growing evidence suggests that oxidative stress (OS) is involved in many aspects of male infertility. The high mitotic rate of germ cells makes male gonads highly susceptible to chemotherapeutic damage. Testis contains polyunsaturated fatty acids in its membrane, making it more sensitive to ROS-induced oxidative stress and subsequent lipid peroxidation, which can cause testicular dysfunction [17].

Cyclophosphamide produces testicular toxicity by inducing spermatotoxicity, oxidative stress and DNA damage. Cyclophosphamide reduced sperm count could be due to interruption in the process of spermatogenesis and damage in germ cell function or epididymal storage. Swelling of the mitochondria can change the protein gradient across the mitochondrial membrane leading to decreased sperm motility. The increase in sperm abnormalities may be attributed to the direct toxic effects of CP on spermatogenesis. DNA damage can also contribute to increased sperm abnormalities [18].

The protection of the structure of DNA is extremely important to transfer genetic information from generation to generation. DNA damage due to genotoxic drugs is an important type of stress to which organisms are exposed during their life. The factors that cause DNA damage can be endogenous and exogenous. Among exogenous sources, alkylating agents come first to cause DNA damage. Alkylating exogenous agents are capable of adding bases to ethyl or methyl groups, get covalently linked to DNA and cause DNA damage, creating an indirect effect of the alkylating agent. Cyclophosphamide (CP), an alkylating agent, causes DNA damage by changing the function of cellular proteins. CP has been shown to induce gene mutations in prokaryotes and eukaryotes, chromosome effects, unscheduled DNA synthesis, and sister chromatid exchange.

Furthermore, cessation of cell growth arrest and DNA damage causing changes in gene expression have been observed due to alkylating agents exposure [19].

In this study, possible DNA damage was measured using the comet assay. The comet assay is currently extensively used in in-vivo and in-vitro studies for the evaluation of the genotoxic potential of a variety of toxic agents such as chemical compounds, ionizing radiation, and UV radiation, as well as the potential of chemical compounds such as fluoroquinolone antibiotics. The increase in DNA damage of cyclophosphamide can be attributed to oxidative stress. It is already accepted that the generation of ROS can lead to oxidative stress that can cause damage [20].

In our study, *Withania somnifera* (WS) was evaluated for its protective effects on cyclophosphamide (CP) induced testicular toxicity and DNA damage Histopathological analysis indicates that CP produces marked testicular toxicity characterized by atrophy of seminiferous tubules with focal hyalinised tubules, necrosis of germ cells and desquamation. Vacuolization and marked reduction of germ cell count with the absence of spermatogenesis were other features of CP group. Our findings are in agreement with *Torabi et al.* [21] WSE when given with CP was protective to all the toxic changes observed in CP.

There is an overall increase in the olive tail moment (OTM) of the CP group whereas there is a decrease in the OTM in group CP + WS and is statistically significant when compared with the control implicating a trend of reduction in DNA damage when compared to the CP group. The other parameters of comet score like the percentage of DNA in tail and tail moment also showed a trend of decrease in CP+WS when compared to the control group. The DNA damage by CP was evident from the increased tail length, olive moment and %DNA in the tail. An increase in oxidative stress may lead to damage to DNA as observed by comet.

In CP-treated rats, testicular toxicity was associated with increased DNA damage and diminished plasma testosterone, showing that spermatogenesis and fertility were impaired. Co-administration of WS significantly improved CP-induced changes in

plasma testosterone, spermatogenesis and fertility, toxic stress, and DNA damage. To explain the protective action of WS, several theories are considered. There is a good possibility that the high amount of antioxidant, free radical scavenging activity and improved testosterone production might have protected spermatozoa against CP-induced toxicity. Another explanation is the androgenic property of *Withania somnifera* which might have a protective effect on spermatozoa during spermatogenesis [22].

Previous studies have reported that *Withania somnifera* can decrease serum FSH level and increases LH level in rats [23]. *Withania somnifera* produces a significant increase in serum testosterone and LH levels and also reduced FSH levels in men, was reported by Ahmed M. K. *et al*. So it is postulated that *Withania somnifera* can improve sperm quality by regulating the reproductive hormone levels by acting on the HPG axis [24]. *Withania somnifera*, an adaptogen, combats stress and can be used as an herbal remedy for stress and infertility. *Withania somnifera* is already patented to improve sperm count in males receiving endocrine-disrupting chemicals [25]. Elumalai Prithiviraj *et al.* also suggests that *Withania somnifera* has a protective effect on cadmium-induced oxidative stress [26]. Another study proved that *Withania somnifera* increased the reproductive hormone level thereby improving sperm quality [27]. The protective effect may be attributed to the potential involvement of the phytochemicals of the extract, as it contains glycosides, flavonoids, saponins, alkaloids, carbohydrates, proteins, and free amino acids [28].

In brief, *Withania somnifera* has a protective effect against testicular toxicity, has good antioxidant activity and its antigenotoxic effects could effectively mobilize DNA repair mechanisms.

CONCLUSION: The findings of our study indicate exposure to cyclophosphamide in rats produces deleterious effects on testicular histology and testosterone and effects DNA. This study suggests the potential effect of *Withania somnifera* against cyclophosphamide-induced testicular toxicity and DNA damage. Further studies may be carried out to understand the exact mechanism of this protective effect.

ACKNOWLEDGEMENT: The authors thank Nitte (Deemed to be University), Mangalore, for financially supporting this research study.

CONFLICT OF INTEREST: The authors declare no conflicts of interest.

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How to cite this article:
Kumar YS, Kumari NS, Sharmila KP and Bhargavan D: Cyclophosphamide induced DNA and testicular damage: protective role of *Withania somnifera*. Int J Pharm Sci & Res 2023; 14(4): 1762-68. doi: 10.13040/IJPSR.0975-8232.14(4).1762-68.