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ORIGINAL ARTICLE

EFFECTS OF *SPIRULINA* AGAINST BPA TOXICITY ON MALE ALBINO WISTAR STRAIN RATS

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Abstract: *Bisphenol-A (BPA) is a colorless, solid, chemical compound primarily used in the manufacturing of various plastics, soluble in most common organic solvents, but has very poor solubility in water. It has been reported that Spirulina has antioxidative, immunomodulatory, and anti-inflammatory properties and works against heavy metal toxicity. It is often used as a food supplement in human, animals, birds and fishes. This study aimed to assess the protective action of the dietary Spirulina against the toxic effects of BPA on testes and bone marrow chromosomes in male rats. Twenty adult Wistar Strain Male Albino Rats were randomly divided into four equal groups (n=5), namely, Group-A, B, C, D and kept under controlled environmental condition (22°C, 12-h light–dark cycle) and food and water were given adlibitum. After 7 days of acclimatization, Group-A treated as control. Rats of Group-B, C and D were given 100 mg BPA/kg body-weight. Additionally, Group-C and D were given 500 and 1000 mg Spirulina/kg body-weight respectively mixed with rat feed. After 24 days all rats were sacrificed. Histological studies were done by routine H-E method and diameter of the seminiferous tubules, its lumen, nucleus of spermatogonia and width of germinal epithelium were measured. Serum testosterone level was measured by rat specific ELISA kit and somatic chromosomes were also prepared from bone marrow by following colchicine–hypotonic-aceto-alcohol fixative–flame drying technique. Results showed that BPA affects the testes and bone marrow chromosome compared to control group. Administration of Spirulina to Group C and D showed gradual recovery of testes architecture and bone marrow chromosome in compare to BPA treated rats. Serum testosterone concentration is lowest in Group-B, whereas Group-C and D have achieved normal testosterone level. Chromosomal abnormalities such as chromatid break, deletion etc. were significantly increased in BPA group compared to group A , C and D (2n=42). Thus, the present study establishes the protective role of Spirulina against the bone marrow chromosomal aberration and testicular damage caused by BPA.*

Keywords: BPA, *Spirulina*, testes, testosterone, bone marrow chromosomal aberrations

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1. INTRODUCTION

There is an increasing concern about the exposure to several endocrine disrupting chemicals (EDCs) which results a number of human reproductive disorders i.e., testicular cancer, precocious puberty, low sperm count, hypospadias, and cryptorchidism etc. One such chemical is Bisphenol A (BPA), a synthetic monomer, which is used in the production of polycarbonate plastics, epoxy resins, food packaging, dental sealant and lacquers for food cans. Humans are exposed to BPA, as it leaches from the inner lining of tin, cans, microwave containers, etc., in contact with acidic or alkaline contents and from dental sealant. The presence of BPA in maternal and fetal plasma, placental tissue and in the milk of lactating mothers, human urine and also in the human serum has also been reported [1]. Higher levels BPA in the urine has been correlated with cardiovascular disease and diabetes and increased risk of miscarriages with abnormal embryonic karyotype [2].

Various animals have been exposed experimentally to BPA to show its multiple effects on the male and female reproductive system. BPA exposure was correlated with the impaired semen quality in brown trout [3] and also in the inhibition of the development of seminiferous tubule and spermatogenesis in male chick [4]. BPA exposed male rats showed decreased sperm count and spermatogenesis [5, 6] and changes in sperm morphology. 2–20 µg/kg/day of BPA caused enlargement of prostate gland, decrease in size of seminal vesicle and epididymis, and daily sperm production in male [7]. BPA exposure has also been associated with poor semen quality and increased sperm DNA damage among men [2].

Spirulina is a microscopic, freshwater single celled alga, which has antioxidative and anti-inflammatory properties. Earlier studies revealed that it can reduce heavy metals toxicity and nephrotoxic substances in the body [8]. *Spirulina* is Also reported to be a rich source of iron, vitamin, alkaloids, terpenoids, steroids, phenols, saponins, flavonoids, tannins, coumarins, quinones, glycosides, etc. [9]. Additionally, it has been reported that *S. platensis* may be associated with modulation of oxidative stress [8].

The present study is an attempt to investigate and evaluate the ameliorating effect of *Spirulina* against BPA induced toxicity in testes and bone marrow chromosomes in male rats.

2. MATERIALS AND METHODS

Animals and experimental design

Twenty adult Wistar Strain Male Albino Rats (average bodyweight 80.35±3.29g) were obtained. All rats were maintained at optimal environmental conditions (22 ºC temperature with a standard dark cycle from 8:00pm to 8:00am). They werefed with a commercial diet and water given *ad libitum*. Rats were acclimatized for one week, then randomly divided into four groups (n=5) as follows-

- **Control group**; Rats received commercial rat feed and water *ad libitum*.
- **BPA group**; Rats were orally administered 100 mg/kg body weight/day of BPA along with rat feed. The current study used a dose of 1/32 of the oral LD50 dose for BPA.
- **BPA+SP_{500}group**; Rats received Spirulina powder at a daily oral dose of 500 mg/kg body weight along with the same dose of BPA.
- **BPA+SP_{1000}group**; Rats received spirulina powder at a daily oral dose of 1000 mg/kg body weight along with the same dose of BPA as before.

All treatments were carried out daily for twenty-four days in order to evaluate their effects.

Table 1: The details of the rats used in the study

	CONTROL	**BPA GROUP**	**BPA+SP_{500}GROUP**	**BPA+SP_{1000}GROUP**
AGE (in week)	4-5	4-5	4-5	4-5
NUMBER	5	5	5	5

Blood samples

Twenty-four hours after the last dose administration, blood was collected and separated clear serum samples were stored at -20°C.

Histological studies

From the sacrificed rats, the testes were collected and kept in 10% formalin, sections were stained by Hematoxylin-Eosin method [10] and observed under microscope (both under 100X and 400X magnification) and micrometric study of seminiferous tubules were done using Scope image-9 software.

Hormonal assessment

Testosterone analysis: Serum testosterone concentration was measured using Rat specific Testosterone Enzyme-Linked Immunosorbent Assay (ELISA) kit [11], (Supplied by G-Biosciences 9800, Page Avenue, St. Louis, MO, USA.)

Chromosomal assessment

Bone marrow cells were collected from femur and chromosome preparation were performed by following the mitotic division inhibition technique described by Chakrabarti *et al.*, and Banerjee and Mallick [12,13]. Only well spread metaphases were analyzed under the Binocular Research Microscope (1000x magnifications).

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM).
Differences between the means were detected by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparisons test using Prism 8.
The level of significance was established at $^{***}p < 0.001$ and $^{****}p < 0.0001$.
The percentage of affected metaphase in the control and treatment series was statistically analyzed using a t-test.

3. RESULTS

Histological Observations

The histological study of testes reveals that in BPA treated group, (Figure 1B and Figure 1F) there are separation of germinal epithelia and obliteration in the wall of some seminiferous tubules.

On the other hand, low dose of *Spirulina* (Figure 1C and 1G) treatment shows significant increase in the germinal cell population with almost normal Sertoli and Leydig cells in seminiferous tubules.

The condition is further revived after high dose of *Spirulina* (Figure -1D and 1H) treatment.

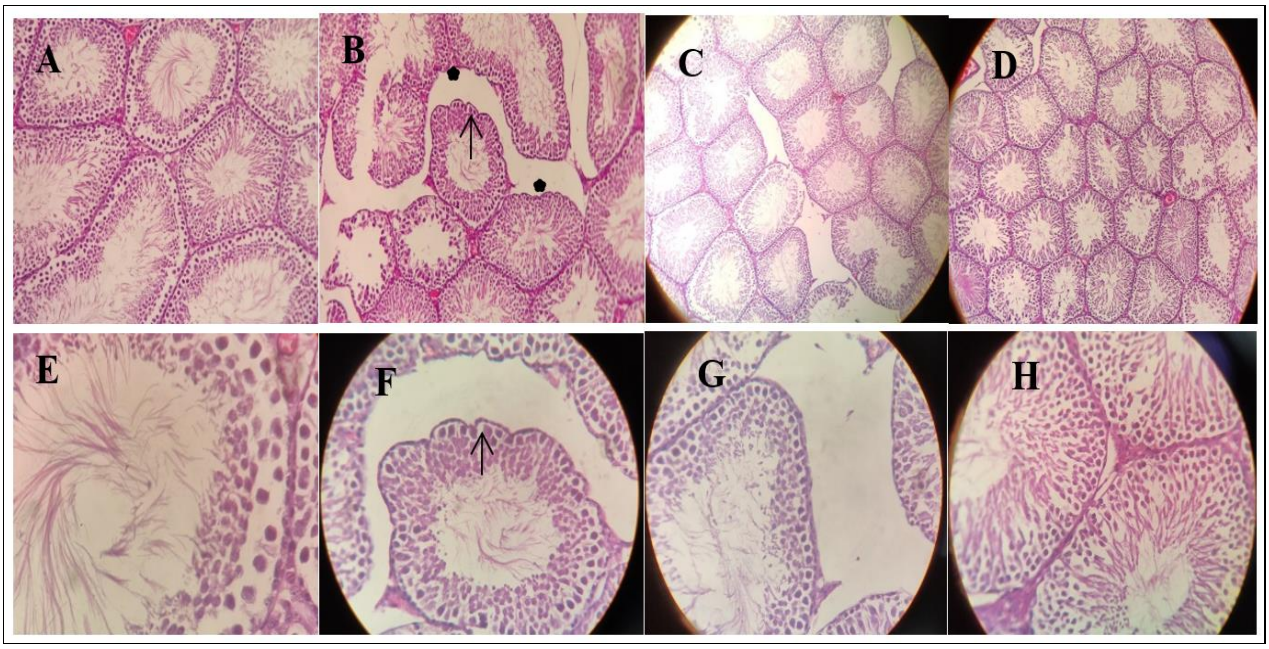

Figure 1: Hematoxylin and Eosin-stained photomicrographs of testis in experimental groups. Rats of control (A: 100× and E: 400×) result normal seminiferous tubules histoarchitectures with active spermatogenesis. The BPA group (B: 100× and F: 400×) exhibits collapsed seminiferous tubules (black arrows), impaired spermatogenesis, edematous fluid accumulation (black asterisks) and germ cells detachment. BPA+SP_{500} co-treated rats show nearly normal testicular histoarchitecture (C: 100× and G: 400×) though collapsed seminiferous tubules also present there. BPA+SP_{1000} co-treated rats exhibit almost normal testicular histoarchitecture (D: 100× and H: 400×) as compared to control.

Table 2: Micrometry study of testes in 400x magnification

PARAMETERES (µm)	GROUPS			
	Control	BPA	BPA+SP_{500}	BPA+SP_{1000}
Diameter of seminiferous tubule	540.7±19.79	476.2±13.88	585.2±14.92	587±36.62
Diameter of lumen	300.7±15.41	246.2±30.19	365.2±7.455	308.9±25.33
Width of germinal epithelium	110.3±10.01	126±10.38	117.5±10.27	134.3±8.08
Diameter of nucleus of spermatogonia	12.71±0.29	12.01±0.54	11.27±0.32	12.07±0.35

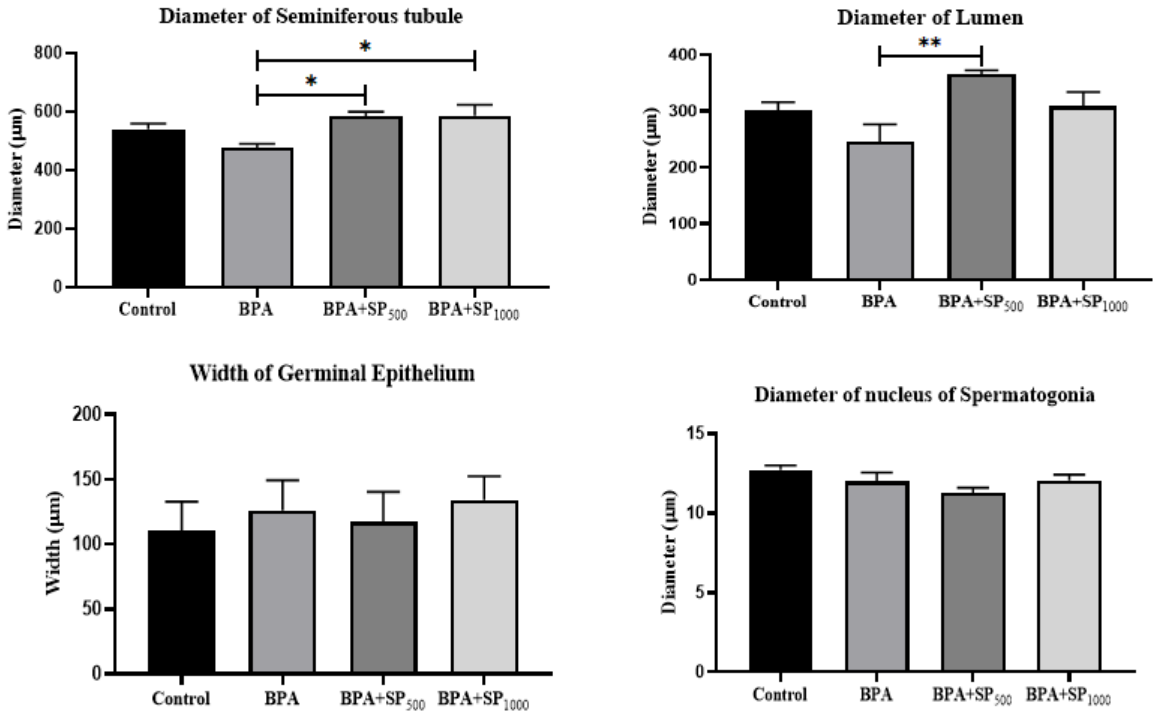

Figure 2: Statistical comparison of histological parameters of testes among different groups is presented here. In this study there are significance difference observed between the diameter of seminiferous tubule of the BPA and BPA+SP_{500} (P=0.02), BPA and BPA+ SP_{1000} (P=0.018). Significant difference was also observed between the diameter of lumen of BPA and BPA+SP_{500} (P=0.006); no significant difference was observed among the other groups.

Serum testosterone concentration

Our study revealedsignificantly lower serum testosterone concentration in BPA group compared to control group ($p < 0.0001$). Gradual increase of testosterone concentration was seen in low and high dose of *Spirulina* treated groups respectively ($p < 0.0001$) when compared tothe BPA group. Though BPA+SP_{1000} group exhibited still significantly lower testosterone level than the control group ($p < 0.0001$) (Figure 3).

Table 3: Serum testosterone concentration (ng/ml) among different experimental groups

PARAMETER	GROUPS			
	Control	BPA	BPA+SP_{500}	BPA+SP_{1000}
Serum testosterone concentration (ng/ml)	7.842 ± 0.003	1.791 ± 0.039	5.670 ± 0.004	6.326 ± 0.001

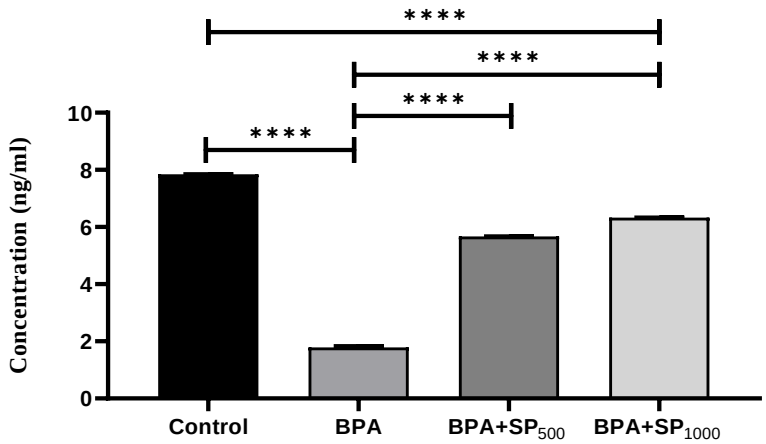

Figure 3: Bar diagrams showing serum testosterone concentrations of different experimental groups. Data are shown as mean ± SEM. One-way ANOVA was used for statistical analysis with Tukey's multiple comparisons test (**P < 0.0001). The significance level was set at P < 0.05.**

Chromosomal assessment

In the present study bone marrow toxicity was analyzed based on chromosome numbers. In control group 21 pairs of chromosomes were found that is the normal chromosomal arrangement of rats (2n = 42). In case of BPA group 2n=36 – 40 chromosomes (i.e. aneuploidy) observed in several locations indicating cytological abnormality. Although again normal chromosomal arrangements (2n =42) were found in low and high dose of *Spirulina* treated groups (BPA+SP_{500} and BPA+SP_{1000}) though they were also exposed to same doses of BPA. (Figure 4).

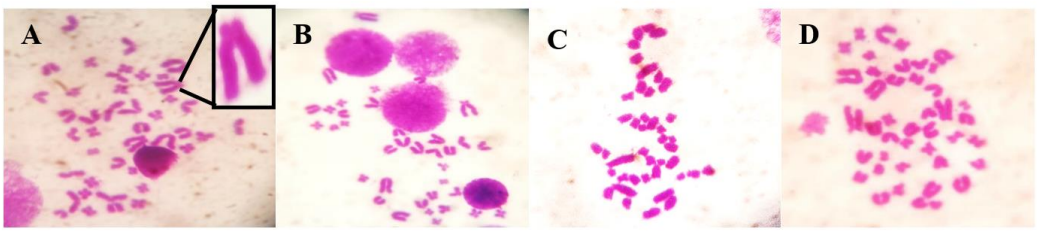

Figure 4. Metaphase figures of chromosomes of bone marrow cells of different experimental groups (1000× magnification); A. Control group, B. BPA group, C. BPA+SP_{500} group, D. BPA+SP_{1000} group.

Table 4. Percentage of affected metaphase in control and treatment series. 75 metaphases studied from 3 specimens (i.e. 3 replications) in each group. Different types of chromosomal aberrations and aneuploidies are found. Affected metaphases are significantly increased in BPA group than BPA +$SP_{500}$and BPA +SP_{1000} group. **P < 0.001; * P < 0.05 when compared to the control value. Values are expressed as mean± SE (n = 3).

Serial No	Experimental Parameters	Percentage of Affected metaphase
1	CONTROL GROUP	5.33 ± 1.33
2	BPA GROUP	14.67 ± 1.33*
3	BPA+SP_{500} GROUP	8.00 ± 2.31
4	BPA+SP_{1000} GROUP	6.67 ± 1.33

4. DISCUSSION

Oxidative stress is a consequence of an imbalance between production of ROS and the body's antioxidant defense capacity [14]. It has been identified as one of the very important factors that affect fertility status. Sperm, like any other aerobic cells, are constantly facing the "oxygen-paradox". Oxygen is essential to sustain life as physiological levels of ROS are necessary to maintain normal cell function and all that is true for sperm as well. However, excessive production of ROS (oxidative stress) is well known to be detrimental to sperm by adversely affecting the quality of sperm DNA [15].

Our studies revealed that BPA causes damage to testicular cells as a potent endocrine disrupter that binds the androgen receptor, acting as an antagonist [16] and affects the major regulatory element of androgen cell signaling which is essential for male reproductive function and development [17]. It has been recently shown that exposure of spermatozoa to BPA *in-vitro* increases ROS [18].

Previous studies have shown that BPA impairs redox homeostasis via increasing of oxidative mediators and by reducing antioxidant enzymes, causing mitochondrial dysfunction, alteration in cell signaling pathways, and induction of apoptosis [19]. This may result the histological changes in testes [20], our histological findings also support this earlier observation. [Fig 1 B and 1F].

In the current study, it has been noticed that *Spirulina* ameliorate the BPA induced toxicity and can reduce the degenerative changes in the testes [Fig 1C. 1D, 1G, 1H] and this may be due to elevated levels of testicular superoxide dismutase (SOD), catalase (CAT), zinc and a decrease level of malondialdehyde (MDA) [21].

Testosterone level also supports our histological findings. [Figure 3]. It can be suggested that *Spirulina* tend to increase Protein Kinase A (PKA)-dependent steroidogenic acute regulatory protein (StAR) expression by decreasing the DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on the X chromosome, gene 1) expression and inhibiting cyclooxygenase (Cox)-2dependent signaling. This contributes to increased testosterone production from Leydig cells [22] that may also help to restore the testicular architecture.

BPA induced oxidative stress in bone marrow cells may be the cause of DNA damage and aneuploidy [23-25].It has been reported that *Spirulina* extract is an important anti-mutagenic agent in rats [26] and is often used as a therapeutic agent. The present chromosomal study on bone marrow cells showed the protective effect of *Spirulina* [Figure 4; Table - 4] against the mutagenic or genotoxic impact of BPA. Therefore, the observations regarding the protective effect of low and high doses of *Spirulina* against BPA (BPA+SP500 and BPA+SP1000) on bone marrow cells of mice are validated by the results of the histopathological structure of the testes in the BPA + *Spirulina*-treated experimental groups. As the findings was observed in some restricted fields of our microscopic views in somatic cells only, so further study is needed to establish *Spirulina* in the field of therapeutics against BPA mutagenicity.

However, till date, different antioxidant approaches have been identified to counteract BPA-induced damage in male reproductive system. So, *Spirulina* rich in antioxidants, vitamins and minerals can be used as a potent therapeutic agent to ameliorate the toxicity induced by BPA. It may be concluded that the protective actions of *Spirulina* against BPA are believed to originate from its free radical scavenging, antioxidant activities, maintenance of antioxidant enzymes and its ability to decrease the inflammatory mediators that are involved in the pathogenesis of BPA induced testicular injury.

Therefore, *Spirulina* represents a potential agent to prevent testicular injury and dysfunction induced by BPA

exposure at a dose specific time dependent manner.

5. ACKNOWLEDGEMENT

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