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Research Article

SUPPRESSION OF MALE REPRODUCTIVE FUNCTION BY BROWN HT IN RAT

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ABSTRACT

Objective: Brown HT is an extensively used food color. Our objective was to evaluate the possible toxic effect of Brown HT on male reproductive functions in rats.

Methods: Brown HT was administered to male rats orally at 100, 200, and 400 mg/kg body weight/day for a period of 30 days. At the end of this period, different types of parameters related to male reproductive physiology and activities of different antioxidant enzymes were measured by spectrophotometric method using standard protocols. The serum hormonal levels were measured by Chemiluminescence Immuno Assay method using the kit.

Results: We have observed significant decrease in mean body weight, gonado somatic index, number of epididymal sperms, percentage of motile sperms, and also decrease in serum level of luteinizing hormone, follicle-stimulating hormone (FSH), and testosterone in a dose dependent manner in Brown HT exposed rats. The activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and glutathione-S-transferase have been decreased and the level of malondialdehyde, a biomarker of lipid peroxidation has been increased significantly in exposed rats. Histopathology of testes also showed degeneration of germ cells in somniferous tubules in Brown HT exposed rats.

Conclusion: According to this result, it is concluded that Brown HT suppresses the male reproductive functions probably by producing oxidative stress in testicular tissues and suppressing the hypothalamus-anterior pituitary-testicular axis in rat.

Keywords: Brown HT, Testes, Sperm count, Luteinizing hormone, Follicle-stimulating hormone, Testosterone, Oxidative stress.

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INTRODUCTION

We understood from several scientific literatures that different types of food additives are used by human beings from a long time approximately since 1500 B.C to conceal the poor quality of spoiled food products. Before, the discovery of the synthetic dyes, people often used different natural substances such as caramel, saffron, and curcumin to color the food products. However, the use of synthetic coaltar dyes, which are much cheaper suppress the use of natural color dyes and provide better coloration. As per Food Adulteration Act, 1954 (PFAA, 1954) in India eight synthetic colors have been permitted to color the different food items considering their low toxicity, namely, carmoisine, erythrosine, tartrazine, indigo, brilliant blue, sunset yellow, ponceau 4R, and fast green [1-3].

Brown HT (Chocolate Brown HT) is a synthetic bis-azo dye, used as food color, consists of reddish-brown powder or granules with molecular formula of $C_{27}H_{18}N_4Na_2O_9S2$. It is the disodium salt of 4, 4'-(2, 4-dihydroxy-5-(hydroxymethyl-1, 3-phenylenebisazo) di (naphthalene-sulfonic acid). It is readily soluble in water and gives the intended color which is very persistent in light and heat [4,5]. Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 followed by the EU Scientific Committee for Food (SCF) in 1984 have suggested that Brown HT can be used as a food additive to produce the intended food color in food industries [6,7]. According to JECFA, 1984 Acceptable Daily Intake (ADI) of Brown HT is about 0–1.5 mg/kg BW/day, while the ADI is about 0–3 mg/kg BW/day according to SCF [8,9].

Although United States and the WHO did not approve Brown HT as a permitted food color in food industries, this dye is abundantly used to enhance the color of several food products such as chocolate cakes, brown bread, biscuits, bakery products, snacks, soft drinks, ice-cream, piddles and sauces, cheese, desserts including flavored milk products, yogurts, jams, soups, fruit products, dessert mixes, canned meat, sugar confectionary, and flour confectionary [5,10-13].

It has been reported that Brown HT produces toxic effects on some health variables in animals due to regular intake with food products. It produces significant reduction in hemoglobin counts, leukocyte counts, red cell counts, hematocrit value, packed cell volume, serum urea level, etc. [14,15]. It produces harmful effects in brain by decreasing the levels of dopamine, norepinephrine, and gamma-aminobutyric acid in some areas of brain [11,14].

Oxidative stress is responsible for many damaging processes in the body which can lead to protein, lipid and DNA damage, tissue damage, inflammation, and subsequent cellular apoptosis, several type chronic diseases such as atherosclerosis, cancer, cardiovascular disease, and neurodegenerative diseases [16]. Spermatogenesis is also susceptible to oxidative stress-induced damage [17]. The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) play a significant role in protecting the spermatogenesis or sperm against peroxidative damage [1]. Any reduction in the activities of antioxidant enzymes due to Brown HT exposure may leads to oxidative stress in reproductive organs. Other synthetic azo dye like tartrazine produces adverse effect on male reproductive function by producing oxidative stress induced damages of testicular tissues, altering the sperm morphology, and sperm count [18,19]. Hence, there may be a possibility to impair the male reproductive functions due to excessive intake of Brown HT. Therefore, the present study was carried out to evaluate if Brown HT exposure could disrupt the structure and function of male reproductive organs and spermatozoa in rats.

METHODS

Reagents and chemicals

Reagents used for this study were of analytical grade. Brown HT was purchased from Red SUN DYE CHEM, India. Trichloroacetic acid, Tris, BSA, Glacial acetic acid, Triton-X-100, Folin–Ciocalteu's phenol reagent, Pyrogallol, Sodium potassium tartrate Thiobarbituric acid, $K_2Cr_2O_7$, 1-chloro-2,4 dinitrobenzene, H_2O_2, sodium azide, etc., were purchased from E-Merck, India. Reduced glutathione, Oxidized glutathione, 5-5′-dithiobis-2-nitrobenzene, and Nicotinamide adenine dinucleotide phosphate, were purchased from Sisco Research Laboratories Private Limited, India.

Animals

Male albino rats weighing between 100-200gm of Charles Foster strain (12–14 weeks old) were used for the experiment. The animals were housed in polypropylene rat cages under suitable environmental condition at a room temperature of 25±2°C with 12:12 h light-dark cycle and adequate ventilation. The animals were fed standard laboratory chow and water daily [20,21]. The animals were nursed as per the recommendation of Animal Ethics Committee of the University of Kalyani based on national guidelines.

Experimental design

After 7 days of acclimatization in laboratory environment, the rats were divided into four groups (control and three treated groups, i.e., Treated-I, Treated-II and Treated-III) including eight animals in each group (Table 1). The treated animals were received Brown HT by oral gavage for a period of 30 days. The doses of Brown HT were selected on basis of the previous studies according to Bawazir, 2012 [11].

Mean body weight

The body weight was calculated on the basis of the weight taken on the day of the application of 1st dose and it was considered as the initial body weight. The body weight taken on the day of sacrifice or 31st day was considered as the final body weight.

Gonadosomatic index (GSI)

After 30 days of exposure duration, the rats were sacrificed; both testes were excised and weighed. GSI was calculated using the following formula:

GSI = weight of testes/body weight × 100 [22,23].

Epididymal sperm count

After 30 days exposure to Brown HT, the rats were sacrificed and the epididymis was carefully separated from the testis. The caudal part severed from its remaining part and weighed [24]. The caudal part of epididymis chopped with the help of sharp blade in 10 ml phosphate buffer saline (PBS, pH-7.4) and spermatozoa were removed from epididymal pieces by vortexing gently in PBS for 10 min. Approximately 20 µl of thoroughly mixed diluted specimen was transferred to each of the Neubauer Counting Chambers of the Hemocytometer and allowed to settle for 10 min in a incubator. After incubation, the number of spermatozoa in the appropriate squares of the hemocytometer was counted using the digital SLR Olympus camera (E-620) fitted with Olympus light microscope (CH20i) at 400× magnification. Total number of sperms was expressed in million/g weight of cauda epididymis [25,26].

Table 1: Animal groups and exposure strategies

Animal groups	Exposure
I	Received distilled water for 30 days (control).
II	Received 100 mg/kg body weight/day of brown HT for 30 days (treated-I).
III	Received 200 mg/kg body weight/day of brown HT for 30 days (treated-II).
IV	Received 400 mg/kg body weight/day of brown HT for 30 days (treated-III).

Sperm motility

Motility of epididymal sperm was evaluated according to the method of Linder *et al.* (1986) and Cooke *et al.* (1991) with slight modifications in both dose- and time-dependent manner (i.e., after 20, 40, and 60, min of sample collection) [27,28]. Epididymal sperm motility was expressed as the percentage of motile sperm of the total sperm counted [25,29,30].

Hormone measurements

After the full-term exposure of the animals to Brown HT for a period of 30 days, the rats were sacrificed by cervical dislocation. Blood was collected through cardiac puncture and centrifuged at 4000 rpm for 15 min at a temperature of 4°C. Serum was collected and stored at a temperature of -20°C for further estimations of reproductive hormones. The serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured by Chemiluminescence Immuno Assay method using the Abbott Barcode System Pack kit (Made in Ireland) [22,31,32].

Biochemical assay

Homogenate preparation

Following sacrifice, both testes were rapidly removed, washed in ice cold saline, dried with a blotting paper and weighed. A 10 g% of testicular tissue homogenate was prepared in 0.1 M phosphate buffer (pH-8.0), with 2 mM EDTA and 0.5% Triton-X-100 by a tissue homogenizer. The testicular tissue homogenate was centrifuged at 2500 rpm for 10 min and supernatant was collected for malondialdehyde (MDA) assay. The pellet was discarded and supernatant was re-centrifuged at 590 g for 10 min. Then, the supernatant was collected and stored at a temperature of -20°C for biochemical antioxidant enzyme assay [33,34].

Estimation of oxidative stress related variables in testicular tissue homogenate

According to the protocol of Marklund and Marklund, (1974) the SOD activity was measured [35]. The activity of CAT was estimated by the protocol of Sinha, (1972) with slight modifications [36]. GR activity was measured according to the method of Staal *et al.*, (1969) [37]. The activity of GPx and Glutathione-S-Transferase (GST) was assayed by the method of Rotruck *et al.*, (1973) and Habig *et al.*, (1973) [38,39]. The amount of MDA, a marker of lipid peroxidation (LPO) was determined according to the protocol of Devasagayam and Tarachand, (1987) [40]. The protein was estimated by the method of Lowry *et al.*, (1951) with bovine serum albumin as the standard [41].

Histological study

After 30 days exposure, the rat was sacrificed, testes were collected, cleaned and dipped in neutral buffered formalin for 48 h for fixation. Then, the tissues were run through several steps according to Bancraft and Gamble (2002) for histological study and at last, the tissue sections were observed under 100× magnifications using the Olympus light microscope (CH20i). Images were obtained by digital SLR Olympus Camera (E-620) [42].

Statistical analysis

Data obtained from the results were presented as mean±SEM. Statistical comparisons between the values obtained in control and Brown HT treated rats were carried out using one-way analysis of variance followed by Student's t-test for paired values; $p \leq 0.05$ was considered as significant.

RESULTS

Effect of Brown HT on the body weight and GSI

The mean body weight of Brown HT exposed groups of rats was decreased significantly in a dose-dependent manner compared to control group of rats for two higher doses (i.e., 200 and 400 mg/kg BW/day) (Fig. 1a). We have also observed a significant decrease in GSI in Brown HT exposed rats for two higher doses (i.e., 200 and 400 mg/kg BW/day) (Fig. 1b).

Effect of Brown HT on epididymal sperm count and sperm motility
We have observed a significant reduction in sperm count of Brown HT exposed groups of rats in comparison with control group of rats in a dose-dependent manner for two higher doses (i.e., 200 and 400 mg/kg BW/day) (Fig. 2a).

We have also found a significant reduction in percentage of sperm motility in exposed rats dose and time dependently (i.e., after 20, 40, and 60 min of sample collection) (Fig. 2b).

Effect of Brown HT on morphology of epididymal sperms
We have observed no significant changes in epididymal sperm morphology in exposed groups of rats in comparison with control group of rats (Fig. 3).

Effect of Brown HT on male reproductive hormone levels
We have found a significant decrease in the levels of some major male reproductive hormones. The levels of LH and testosterone in the serum of Brown HT exposed groups of rats have been decreased significantly in a dose response manner (Fig. 4). We have also found significant reduction in serum level of FSH only in higher dose exposed rats in comparison with control rats.

Effect of Brown HT on antioxidant defenses in testes.
The activities of different antioxidant enzymes such as SOD, CAT, GPx, GR, and GST in testicular tissue homogenate have been decreased significantly in a dose-dependent manner in Brown HT exposed groups of rats compared to control group of rats. Further we have found a significant increase in the level of MDA in testicular tissue homogenate in Brown HT exposed groups of rats compared to testes of control rats (Fig. 5).

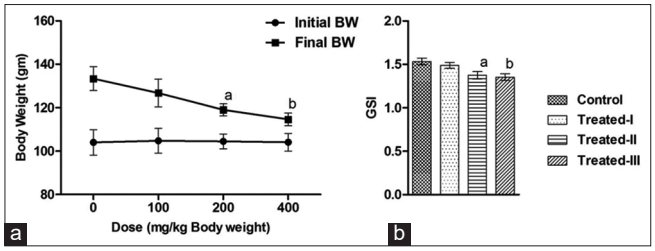

Fig. 1: Graphical representation showing the (a) mean body weight of control and Brown HT exposed rats and (b) the Gonado Somatic Index of control and Brown HT exposed rats. Data are expressed as mean±SEM. [a]p<0.05, [b]p<0.01, [c]p<0.001 versus control, (n=8)

Histopathological findings
Histological analysis of the testes of control rats showed normal morphological appearance of seminiferous tubule with stratified epithelium. However, histological analysis of exposed groups of rats showed structural alterations in the cellular structure of seminiferous tubules with sloughing of germ cells from the basement membrane and gross impairment in the orientation of the germ cells in seminiferous tubules in comparison with control histological structure of seminiferous tubules (Fig. 6). Besides, the number of the germ cells in seminiferous tubules has been decreased. Further the diameter of the lumen of seminiferous tubules in Brown HT exposed rats has been increased in a dose response manner compared to control rats (Table 2).

DISCUSSION

Brown HT is a synthetic food color and has been used heedlessly in Indian food industries [6,7,12]. From the evidences, it has been clear that this compound produces toxic effects on different organs of the body such as brain, liver, heart, blood, and kidney. Hence, the aim of the present study was to evaluate the probable toxic effects of Brown HT on the male reproductive functions in rat model to understand the probable mechanism of intoxication of Brown HT in male reproductive function in humans.

GSI is an index used to measure sexual maturity in relation to the sexual development of testes. GSI predicts the rates of sperm production as well as sperm function in a given species [43,44]. GSI depends on size and mass of testes. In our study, GSI has been decreased in Brown HT exposed rats. Our result indicates that Brown HT reduced the testicular mass of the exposed groups of rats in comparison to control rats. From the earlier study, it is reported that there is a positive correlation between testicular mass and sperm production [45,46]. Hence, our result suggests low sperm production efficiency.

Sperm concentration in semen, morphology of the sperm and sperm motility are three primary indices which are being considered to

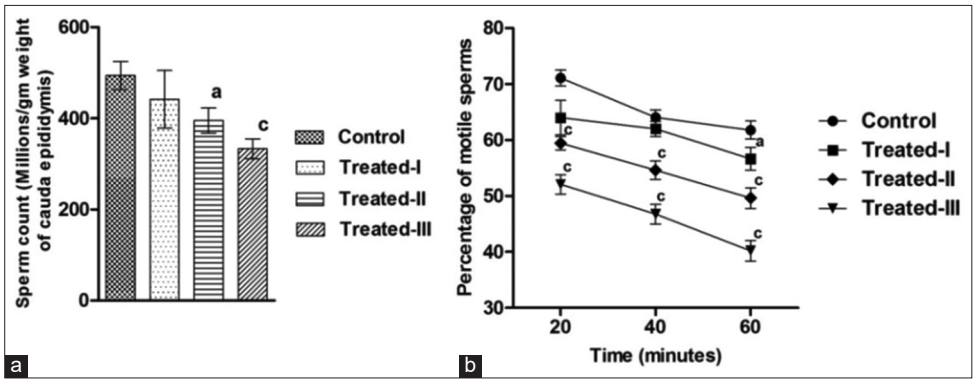

Fig. 2: Graphical representation showing the (a) sperm count (millions/g weight of cauda epididymis) and (b) percentage of motile sperm after 20, 40 and 60 min of sample collection in control and Brown HT exposed rats. Data are expressed as mean±SEM. [a]p<0.05, [b]p<0.01, [c]p<0.001 versus control, (n=8)

Table 2: The area of seminiferous tubule (mm^2) and area of lumen (mm^2) in control and brown HT treated rats

Parameters	Control	Treated-I	Treated-II	Treated-III
Area of semniferous tubule (mm^2)	0.053±0.003	0.051±0.002	0.055±0.002	0.057±0.004
Area of lumen (mm^2)	0.009±0.001	0.013±0.001[b]	0.018±0.001[c]	0.023±0.003[c]

Data are expressed as mean ± SEM. [b]p<0.01, [c]p<0.001 vs. control, (n=8).The area were measured using the application Image J-1.4.3.67.

evaluate the male fertility and reproductive system toxicity [29,47-49]. The epididymal sperm count and the percentage of motile sperm have been determined in exposed groups of rats to ascertain the probable role of Brown HT in the suppression of spermatogenesis. We have found significant reduction in sperm count of Brown HT exposed groups of rats in comparison with control group of rats in a dose-dependent manner for two higher doses (i.e., 200 and 400 mg/kg BW/day) and significant reduction in percentage of sperm motility in Brown HT exposed rats compared to control group of rats has been observed both in dose- and time-dependently. The decrease in percentage of sperm count might be due to Brown HT induced damages of seminiferous tubules as revealed by the decrease in testicular mass. The result indicates that Brown HT not only inhibits the process of spermatogenesis, but also inhibits the process of acquisition of motility by the spermatozoa.

FSH, LH, and testosterone have roles in the maintenance of male reproductive functions and therefore determining levels of these hormones are important in reproductive toxicity studies [50]. Testosterone is the principal sex hormone of the male individuals. It controls the process of spermatogenesis by promoting the production of spermatozoa from spermatogonia through inducing meiotic division of primary spermatocytes into spermatids; and also the process of maturation of spermatid into spermatozoa within the seminiferous tubules [51,52]. Testosterone is released from the interstitial cells of Leydig in the testes under the influence of LH secreted from anterior pituitary. FSH secreted from the anterior pituitary regulates the production of spermatozoa in Sertoli cells [31,53]. The hypothalamic-pituitary-gonadal axis can be affected by many factors. Different types of chemicals including drugs suppress the spermatogenesis and decrease the fertility or even lead to infertility in both males and females by disrupting the normal function of this axis [54]. To examine the involvement of FSH, LH, and testosterone in Brown HT induced suppression of spermatogenesis, the serum level of FSH, LH, and testosterone in Brown HT exposed rats has been determined. We have found significant reduction in serum levels of LH, FSH, and testosterone in exposed groups of rats compared to control group of rats. This result also supported by the results of Abbas and Al-Hamadawi, 2019 [55]. Our results suggest that Brown HT suppresses the process of spermatogenesis and thus, fertility probably by inhibiting the release of FSH and LH from the anterior pituitary and testosterone from the testicular Leydig cells. To find out the effects of Brown HT on sperm counts and acquisition of motility by the spermatozoa during maturation, the sperm count and percentage of motile sperms have been performed in Brown HT exposed groups of rats. We have observed significant reduction in the total numbers of sperms and numbers of motile epididymal sperms in exposed rats. This result indicates that Brown HT probably impairs the acquisition of motility by the spermatozoa during maturation process in the epididymis. The impairment of the sperm maturation and significant reduction in the number of mature spermatozoa in exposed groups of rats might be due to Brown HT induced suppression of the secretion of FSH and LH from anterior pituitary and testosterone from interstitial cells of Leydig. As FSH and LH are released from gonadotroph cells of the anterior pituitary under the tropic action of GnRH, the releasing hormone released from hypothalamus there is a possibility of the suppression of the release of GnRH under the inhibitory influence of Brown HT. In other words, Brown HT impairs spermatogenesis and sperm maturation probably by suppressing the hypothalamus-anterior pituitary-testicular axis.

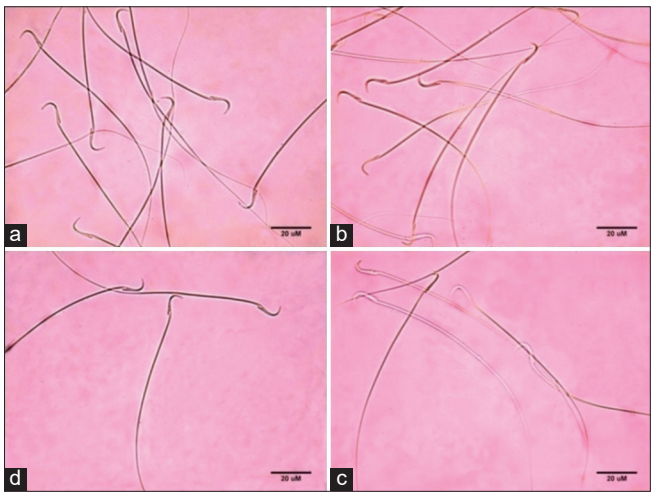

Fig. 3: Photomicrographs (400× magnification) showing the epididymal sperm morphology in control and Brown HT treated groups of rats. (a) Control, (b) treated-I (100 mg/kg BW/day), (c) treated-II (200 mg/kg BW/day), and (d) treated-III (400 mg/kg BW/day). Images were taken with digital SLR Olympus camera (E-620) fitted with Olympus light microscope (CH20i)

From the previous report, it has been suggested that oxidative stress is an important factor in the decrease of semen quality and male infertility [56,57]. To find out the involvement of oxidative stress in Brown HT induced suppression on male reproductive function, different oxidative stress variables of testicular tissue have been studied in Brown HT exposed rats. We have observed significant decrease in the activities of SOD, CAT, GPx, GR, and GST in testicular tissue homogenates of exposed groups of rats compared to control group of rats. We have also found significant increase in the level of MDA in testicular tissue homogenate of Brown HT exposed rats. Brown HT probably induces the accumulation of reactive oxygen species (ROS), namely, hydroxyl radical ($HO^{\bullet}$), Superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydroperoxyl ($HO_2^{\bullet}$), and hydrogen peroxide (H_2O_2) in the cells of testes by inhibiting the activities of various antioxidant enzymes as observed in this study. The accumulated ROS, in turn, may induce the damage of cellular macromolecules like proteins, lipids and DNA. There is a possibility of the disruption of cell signaling mechanisms in testicular cells mediated by male reproductive hormones. The damage of the lipid macromolecules in the testicular cells has been revealed by

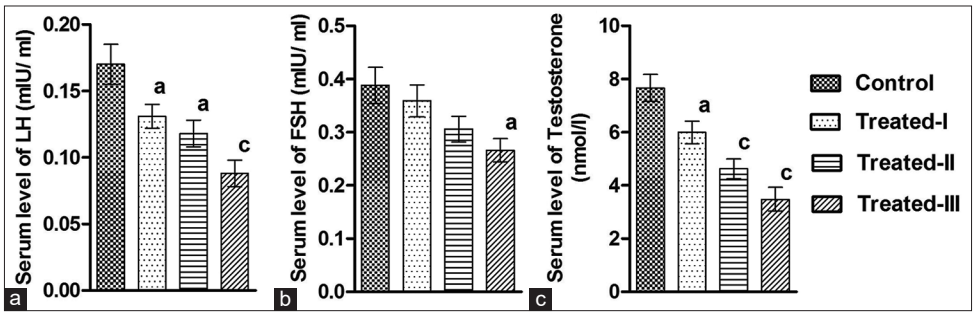

Fig. 4: The changes in the concentration of male reproductive hormones in 3 Brown HT treated groups of rats compared to the control group of rats. (a) Serum level of Luteinizing Hormone; (b) serum level of Follicle Stimulating Hormone; and (c) serum level of Testosterone. Data are expressed as mean±SEM. [a]p<0.05, [b]p<0.01, [c]p<0.001 versus control, (n=8)

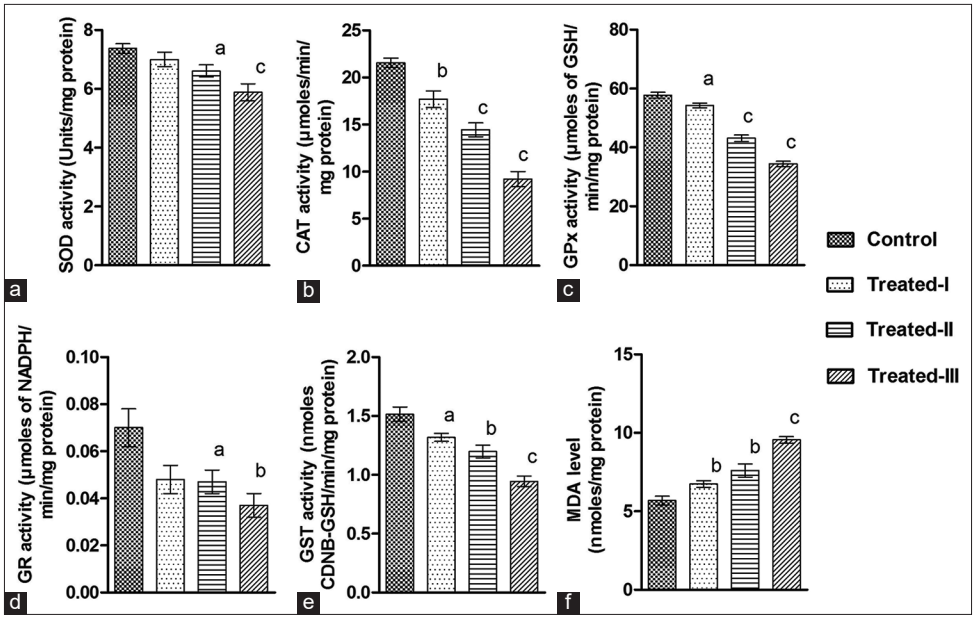

Fig. 5: Graphical representations showing the activities of antioxidant enzymes: (a) Superoxide dismutase, (b) Catalase, (c) glutathione peroxidase, (d) glutathione reductase, (e) glutathione-s-transferase and serum level of, (f) malondialdehyde in testicular tissue homogenate of control and Brown HT exposed rats. Data are expressed as mean±SEM. [a]p<0.05, [b]p<0.01, [c]p<0.001 versus control, (n=8)

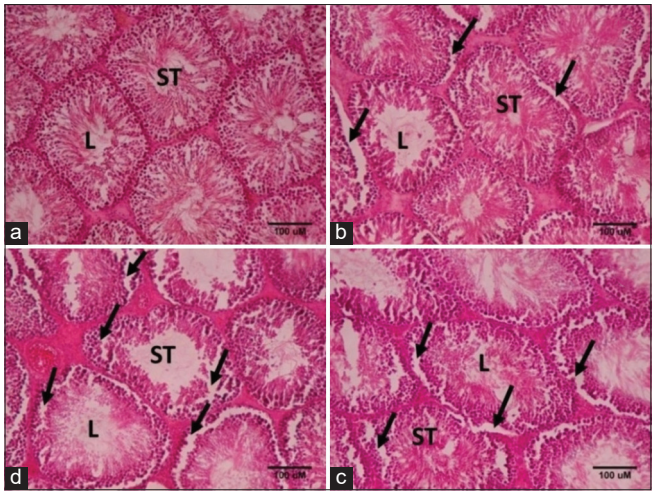

Fig. 6: Photomicrographs (100× magnification) showing the histological alterations (Stained with H and E) in testes in Brown HT exposed and control groups of rats. (a) Control, (b) Treated-I (100 mg/kg BW/day), (c) Treated-II (200 mg/kg BW/day) and (d) Treated-III (400 mg/kg BW/day). Arrow heads indicate the sloughing of germ cells from basement membrane. ST represents seminiferous tubule, L represents lumen of seminiferous tubule. Images were taken with digital SLR Olympus camera (E-620) fitted with Olympus light microscope (CH20i)

the increase in the level of MDA, the marker (end product) of LPO, in testicular tissue homogenate. From the results we could hypothesize that Brown HT may induce the damage of testicular tissue probably by inducing oxidative stress in testicular cells.

Damage of the testicular tissue due to Brown HT induced oxidative stress might be one of the reasons for the suppression of the male reproductive functions by the Brown HT. To examine the involvement of Brown HT in testicular tissue damage, the histo-anatomical structure of testes in Brown HT exposed rats have been studied by histological techniques. We have found significant structural changes in eosin-hematoxylin stained transverse sections of testis as observed and subsequently photomicrographed under light microscope. A significant sloughing of germinal epithelium from the basement membrane and gross impairment in the orientation of germ cells in seminiferous tubules has been observed in exposed rats. We have also observed significant reduction in the number of stratified germinal epithelial cells in the seminiferous tubules. This result suggests that Brown HT might induce the changes in the normal orientation of cells in the seminiferous tubules and decrease the number of germinal epithelial cells probably by causing oxidative stress induced damages.

Considering all the results in this study it may be suggested that Brown HT suppresses the male reproductive functions in rat probably by damaging testicular tissues through oxidative stress, reducing the plasma level of FSH and LH, released from anterior pituitary, inhibiting the process of sperm production by spermatogenesis, decreasing the percentage of motile sperms and reducing the secretion of testosterone from the interstitial cells of Leydig. The results obtained from the studies in rat suggest that Brown HT may be considered as potential human toxicant and it could suppress the function of male reproductive system in human.

CONCLUSION

According to the results of our study, it can be concluded that Brown HT suppresses the male reproductive function by producing oxidative stress in testicular tissue and reducing the serum hormonal levels, that is, LH, FSH, and testosterone or suppressing the hypothalamus-anterior pituitary-testicular axis in rat (Fig 7).

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AUTHORS' CONTRIBUTION

AK, the main researcher involved in study design, experimental study, data analyzing, and manuscript preparation. GP, the corresponding

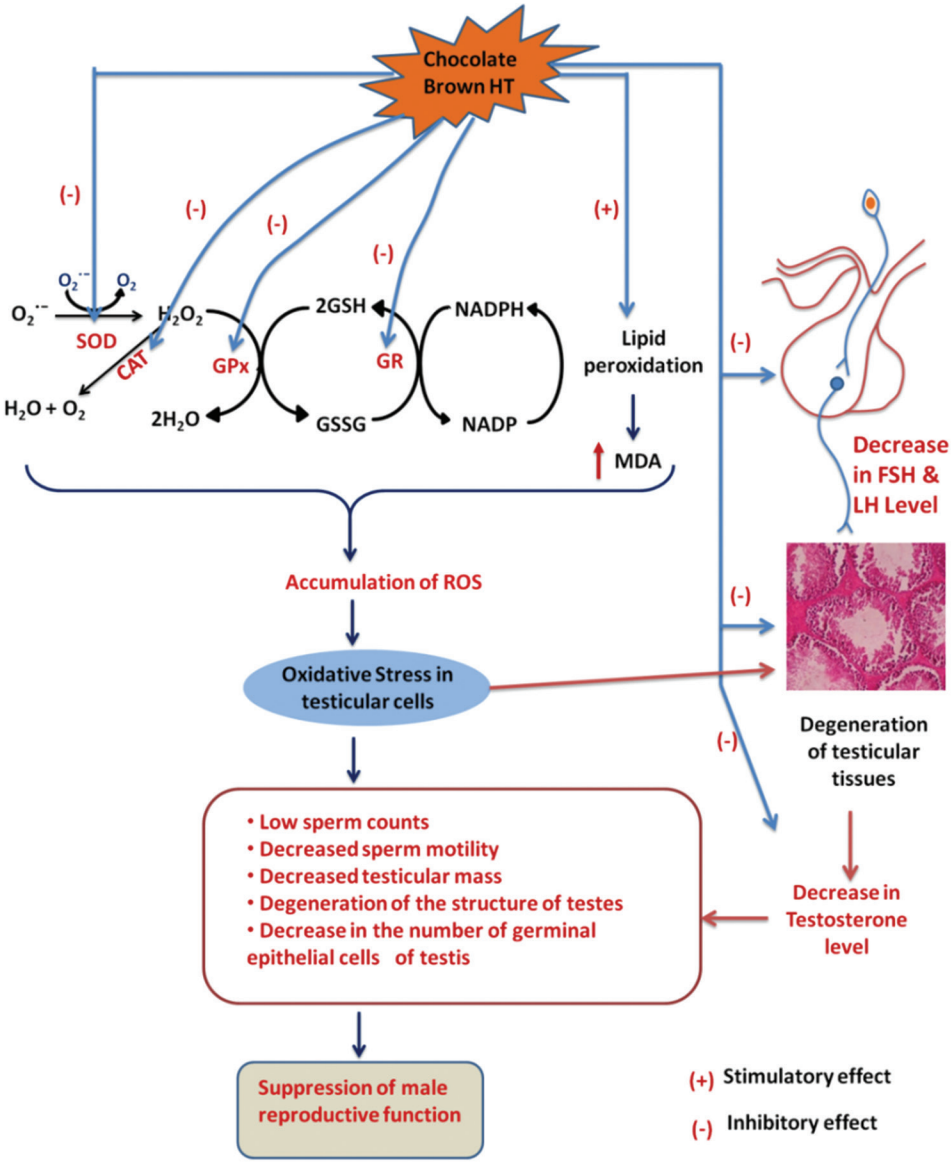

Fig. 7: Probable mechanism of action of Brown HT in the suppression of male reproductive function in rat

author involved in study design, manuscript preparation, and correction. The other coauthors involved as a technical assistant.

CONFLICT OF INTEREST

We declare no potential conflicts of interests in this research studies.

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