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Impairment of uterine wall structure by Chocolate Brown HT in rats

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Abstract

Chocolate Brown HT, the bis-azo dye which is extensively used to color different types of foods. We aimed to evaluate the role of Chocolate Brown HT on the functions of the uterus. Studies were carried out on adult female albino rats of the Charles Foster strain. For this study rats were randomly separated into four groups: one was the control group, and the other three groups were exposed to three different effective dosages (100mg/kg body weight/day, 200mg/kg body weight /day, and 400mg/kg body weight /day) of Chocolate Brown HT for 30 days. After the termination of this period, alterations to body weight, the weight of uterus, activities of different antioxidant enzymes, and histomorphology of uterine wall structure were measured. We have observed a significant decrease in mean body weight and weight of uterus in exposed rats. The activities of different antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-s-transferase (GST), and glutathione peroxidase (GPx) were decreased and the level of malondialdehyde (MDA), a biomarker of lipid peroxidation was increased significantly in Chocolate Brown HT exposed rats compared to control group of rats in a dose-dependent manner. From the histomorphological study, significant degeneration and lesions in the wall structure of the uterus have also been detected in Chocolate Brown HT exposed rats. From our study, it may be concluded that Chocolate Brown HT impairs the function of the uterus probably by producing oxidative stress-induced damages of uterine tissues.

Keywords Chocolate Brown HT, uterus, oxidative stress, lipid peroxidation, histopathological study of uterus.

Introduction

The visual aspect is the key factor for the selection of food products by consumers. Different types of coloring agents are widely used in current times as food additives for enhancing product quality, taste, texture, and color of food to make it more appealing. But the substances used as food additives may be toxic, causing harmful effects on human beings. It has been reported that some food additives are carcinogenic or even teratogenic (Inetianbor et al., 2015; Shubik, 1975; PFAA, 1954).

Aesthetically pleasing foods are consumed more presumably and thus the probabilities of storing toxic colored substances are increased in the body. Chocolate Brown HT, also a synthetic coal tar bis-azo dye, extensively used as food additives, consists of reddish-brown powder or granules. It is the disodium salt of 4, 4'-(2, 4-dihydroxy-5-(hydroxymethyl)-1, 3-phenylenebisazo) di (naphthalene-sulphonic acid). It is readily soluble in water and gives the intended color which is

very persistent in light and heat (Leo and Fide, 2012; Hong et al., 2016). According to EU Scientific Committee for Food (SCF), 1984 the Acceptable Daily Intake (ADI) of Chocolate Brown HT is about 0-3 mg/kg BW/ day (SCF, 1984; EFSA, 2010). Ignoring the probable human health hazards due to Chocolate Brown HT intoxication in different tissue cells of the body, Chocolate Brown HT is used heedlessly in food industries to impart color in different food products like ice-cream, candies, jams, dessert mixes, nonalcoholic flavored drinks, chocolate cakes, biscuits, soft drinks, pickles and sauces, bakery products, milk, cheeses, yogurts, sugar confectionery, and flour confectionery, etc.(U.S. Food & Drug Administration, 1999).

Different types of toxic effects have been found in previous reports due to regular intake of Chocolate Brown HT in animals. It produces a significant reduction in hemoglobin counts, leucocyte counts, red cell counts, hematocrit value and

serum urea level, etc (Drake et al., 1978; Hall et al. 1966; Chambers et al., 1966). It produces harmful effects in the brain by decreasing the levels of dopamine, norepinephrine, and gamma-aminobutyric acid (Bawazir, 2012). It suppresses the function of the female reproductive system and impairs the male reproductive system by reducing the serum levels of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (Khatun et al., 2017; Abbas et al., 2019).

Several pathological changes in the female reproductive tract related to oocyte maturation, steroidogenesis, implantation of a fertilized ovum, and embryo development are promoted by oxidative stress. Oxidative stress is a consequence of an imbalance in the number of reactive oxygen species (ROS), commonly called free radicals. This imbalance may be caused by several factors like inadequate nutrition, reduced antioxidant activity, and animal retention in stressful conditions related to higher ROS production (Silveira, 2018). Any reduction in the antioxidant enzyme activities because of Chocolate Brown HT exposure may induce oxidative stress in reproductive organs. Therefore, the present study was designed to evaluate the role of Chocolate Brown HT on the functions of antioxidant enzymes in uterine smooth muscles.

Materials and Methods

Reagents and chemicals

All the chemicals and reagents were of analytical grade. Chocolate Brown HT was purchased from Red SUN DYE CHEM, India. Eosin and hematoxylin, NaOH, NaCl, CuSO₄,

Tris, BSA, HCl, K₂Cr₂O₇, trichloroacetic acid (TCA), thiobarbituric acid (TBA), glacial acetic acid, triton-X-100, ethylene diamine tetraacetic acid (EDTA), folic ciocalteau’s phenol reagent, sodium potassium tartrate, pyrogallol, 1-chloro-2,4 dinitrobenzene (CDNB), NaH₂PO₄, Na₂HPO₄, KH₂PO₄, K₂HPO₄, H₂O₂, and sodium azide, etc. were purchased from E-Merck, India. 5-5’-dithiobis-2-nitrobenzene (DTNB), oxidized glutathione, reduced glutathione and nicotinamide adenine dinucleotide phosphate (NADPH.Na₄) were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd., India.

Animals

Studies were carried out on adult female albino rats of Charles Foster strain weighing about 100-120 gm (12-14 weeks old). Polypropylene cages were used for nursing the animals. They were kept under suitable environmental conditions at a temperature of 25 ± 2°C in the equal day-night cycle (12L: 12N). Standard laboratory chow with adequate carbohydrate and protein and water were fed to the animals. They were maintained as per the recommendation of the Kalyani University Animal Ethics Committee.

Animal grouping and exposure strategies

After one week of acclimatization in a laboratory environment, the rats were randomly separated into four groups (Control and 3 treated groups i.e. Treated-I, Treated-II, and Treated-III). Each group contains eight animals for this chronic study (Table 1). Animals of exposed groups were received Chocolate Brown HT by oral gavage for 30 days.

Table 1. Showing 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 Chocolate Brown HT for 30 days (Treated-I).
III	Received 200 mg/kg body weight/day of Chocolate Brown HT for 30 days (Treated-II).
IV	Received 400 mg/kg body weight/day of Chocolate Brown HT for 30 days (Treated-III).

Measurement of the weight of the body and uterus

A sensitive balance (Wensar- electronic balance, India) was used for bodyweight measurement of rats for a period of 30days exposure duration in every 10 alternative days. The weight taken on the day of the application of 1st dose was considered as the initial body weight. The body weight measured on the 31st day or day of sacrifice was considered as the final body weight. For the measurement of absolute organ weight, the uterus was collected after the rat was

sacrificed and weighed (Mondal et al., 2014). For calculation of relative organ weight, the following formula was used-

$$\text{Relative Organ weight} = \frac{\text{Absolute Organ Weight (gm)}}{\text{Body Weight of rat on sacrificed day (gm)}} \times 100$$

Preparation of uterine tissue homogenate

After 30days of exposure, the animals were sacrificed by cervical dislocation, and the uterus was removed. After the removal of the uterus, it was washed in saline and dried with blotting paper. For 2gm % of uterine tissue homogenate preparation, we used 0.1 M phosphate buffer (pH-8.0), 2 mM EDTA and 0.5% Triton X-100 and mixed well by a tissue homogenizer. At 8000 rpm the uterine tissue homogenate was centrifuged for 10 minutes. After the collection of supernatant, it was recentrifuged at 12,000 rpm for 10minutes. At last, after collection of the supernatant, it was stored at a temperature of -20 °C for further biochemical antioxidant enzyme assay (Mondal et al., 2014).

Determination of antioxidant enzyme activities in uterine tissue homogenates

Superoxide dismutase (SOD) activity was measured as per the protocol of Marklund and Marklund, 1974. U/mg protein was used for the expression of SOD activity (Marklund and Marklund, 1974). The activity of catalase (CAT) was estimated by the protocol of Sinha, 1972 with slight modifications, and for the expression of the activity of catalase enzyme, we used μ moles of hydrogen peroxide consumed/min/mg protein (Sinha, 1972). According to the protocol of Staal et al., 1969 the activity of glutathione reductase (GR) was determined, and the activity of GR was expressed as μ moles of NADPH oxidized/min/ mg protein (Staal et al., 1969). The activity of glutathione peroxidase (GPx) was determined by the protocol of Rotruck et al., 1973. The activity of GPx was expressed as μ moles of GSH consumed/min/ mg protein (Rotruck et al., 1973). The activity of glutathione-S-transferase (GST) was analyzed by the procedure of Habig et al., 1973 (Habig et al., 1973). The malondialdehyde (MDA) level which is a marker of lipid peroxidation (LPO) was tested according to Devasagayam and Tarachand protocol and the activity of MDA was expressed as nmole/mg protein (Devasagayam and Tarachand, 1987). The protein was tested following the procedure of Lowry et al., 1951 (Lowry et al., 1951).

Histological study

At the last of exposure duration, the rats were sacrificed; the uterus was collected, cleaned, and dipped in neutral buffered formalin (NBF) for 48 hours for fixation. Then, the uterine portion was run through a bath of graded ethanol to displace the water and finally embedded in paraffin (56°C-58°C) impregnated blocks. The uterine segment was sectioned about 5 μ m thick using a microtome and placed on slides. After xylene treatment uterine sections were washed with graded ethanol. After that, the slides were stained with hematoxylin and eosin and dehydrated with graded ethanol. At last, DPX was used for mounting the tissue sections after clearing with xylene and observed under 100X magnifications using the Olympus light microscope (CH20i) (Bancroft and Gamble, 2002).

Statistical analysis

All the data were calculated and expressed as mean \pm SEM. One-way ANOVA and Student 's t-test were used for statistical comparisons of the values get in control and Chocolate Brown HT exposed rats for paired values; $p \leq 0.05$ was considered as significant.

Result

Chocolate Brown HT exposure on mean body weight and uterine weight in rats:

A significant decrease has been found in mean body weight (Fig.1) of Chocolate Brown HT exposed rats for two higher dosages (i.e. 200mg/kg BW/day and 400mg/kg BW/day), and we have also observed that both absolute and relative weight of uterus were reduced significantly in Chocolate Brown HT exposed rats in comparison with control rats dose-dependently (Table 2).

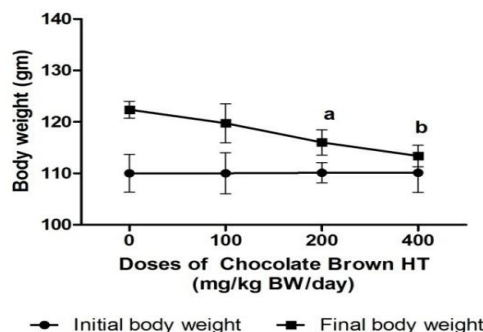


Fig. 1 Graphical representation showing the body weight of control and Chocolate Brown HT exposed rats. Mean \pm SEM is used for data representation. ^a $p < 0.05$, ^b $p < 0.01$ vs. control, (n=8).

Table 2. Showing the absolute and relative weight of uterus in control and Chocolate Brown HT exposed rats. Mean \pm SEM is used for data expression. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. control, (n=8).

	Weight of uterus (gm)	
	Absolute Weight (gm)	Relative Weight (%)
Control	0.316 \pm 0.022	0.259 \pm 0.018
Treated-I	0.251 \pm 0.014 ^a	0.210 \pm 0.012 ^a
Treated -II	0.219 \pm 0.013 ^b	0.188 \pm 0.011 ^b
Treated -III	0.193 \pm 0.010 ^c	0.169 \pm 0.008 ^c

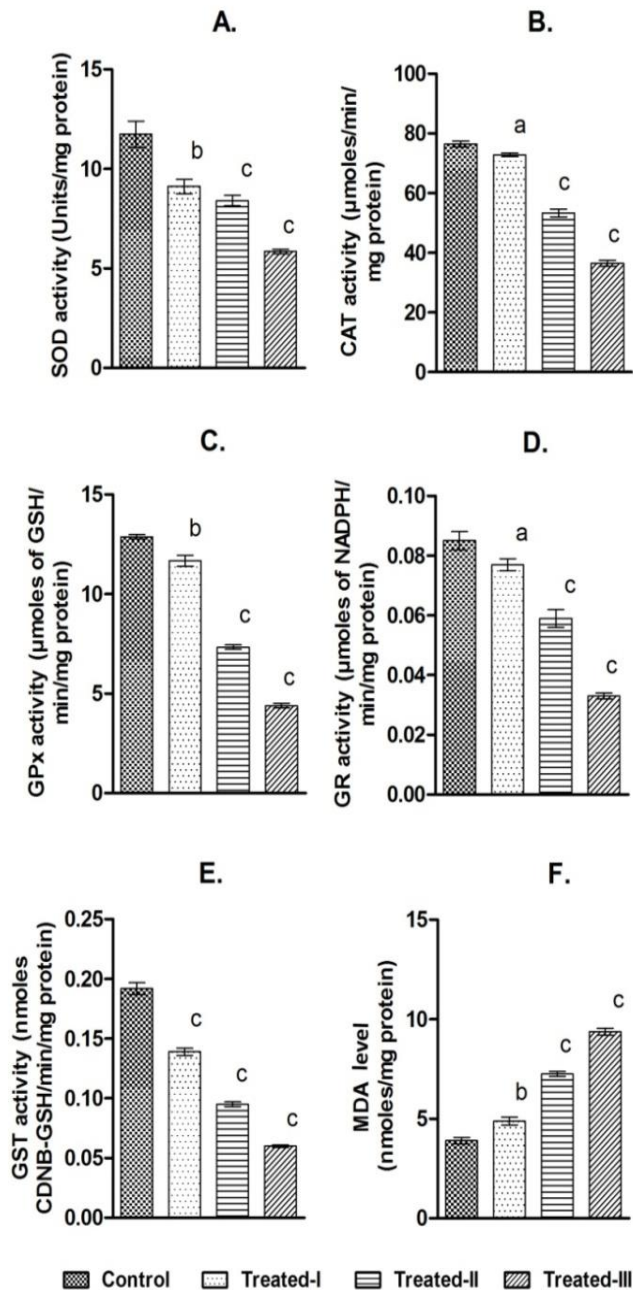


Fig. 2 Graphical representations showing the activities of antioxidant enzymes: (A) Superoxide dismutase (SOD), (B) Catalase (CAT), (C) Glutathione Peroxidase (GPx), (D) Glutathione Reductase (GR), (E) Glutathione-S-Transferase (GST) and serum level of (F) Malondialdehyde (MDA) in uterine tissue homogenate of control and Chocolate Brown HT exposed groups of rats. For data representation, we used mean \pm SEM. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. control, (n=8).

The antioxidant enzyme activities in uterine tissue homogenate due to Chocolate Brown HT exposure

We have observed a significant change in the antioxidant enzyme activities in uterine tissue homogenate in Chocolate Brown HT exposed rats. The activities of SOD, CAT, GPx, GR, and GST in uterine tissue homogenates were decreased significantly in a dose-dependent manner in Chocolate Brown HT exposed groups of rats compared to the control group of rats. We have also found that the production of MDA, a biomarker of lipid peroxidation (LPO) increased significantly in a dose-dependent manner in exposed groups of rats compared to control rats. This suggests that Chocolate Brown HT may damage the uterine tissues by promoting LPO in the biological membranes (Fig. 2).

Histological study of uterine wall structure after Chocolate Brown HT exposure

As far as histopathological study is concerned we have found a significant change in the wall structure of the uterus dose-dependently in Chocolate Brown HT exposed groups of rats compared to control rats. Significant degeneration and lesions in the wall structure of the uterus have also been detected in Chocolate Brown HT exposed rats. Necrosis of the epithelium of endometrial glands was also observed (Fig. 3).

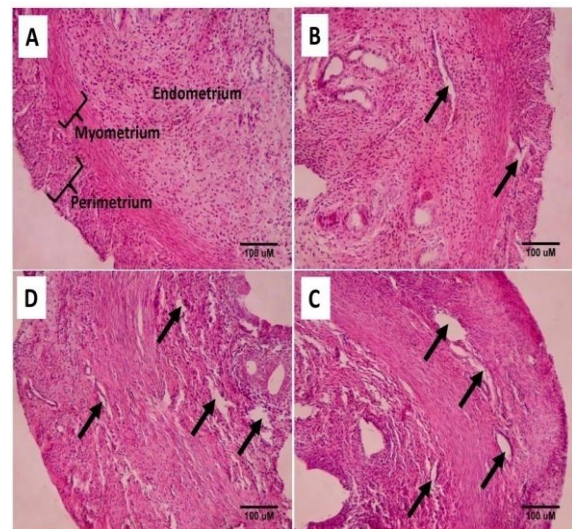


Fig. 3 Photomicrographs (100X magnification) showing the histological alterations (stained with H&E) in the wall structure of uterus in Chocolate Brown HT exposed and control groups of rats. A: control, B: Treated-I (100mg/kg BW/day), C: Treated-II (200mg/kg BW/day) and D: Treated-III (400mg/kg BW/day). Arrow heads indicate the lesions and degenerations in the wall structure of the uterus. For image capturing we were used a digital SLR Olympus camera (E-620) which is fitted with Olympus light microscope (CH20i).

Discussion

The present study was aimed to evaluate the role of Chocolate Brown HT on the functions of the uterus in rats. For this study, we have observed the effect of Chocolate Brown HT on the total body weight and weight of the uterus. A significant decrease has been found in mean body weight (Fig.1) of Chocolate Brown HT exposed rats for two higher dosages (i.e. 200mg/kg BW/day and 400mg/kg BW/day), and we have also observed that both absolute and relative weight of uterus were reduced significantly in Chocolate Brown HT exposed groups of rats compared to control rats in a dose-dependent manner. From the result, it can be recommended that Chocolate Brown HT hampers the growth and proliferation of the uterus.

The molecules with one or more unpaired electrons are called highly unstable free radicals. These unstable radicals easily react to each other and cause the formation of reactive oxygen species (ROS) such as hydroxyl radical (HO[•]), superoxide radical (O₂^{•-}), singlet oxygen (¹O₂), hydroperoxyl (HO₂[•]), hydrogen peroxide (H₂O₂), etc (Bedard et al., 2007; Orient et al., 2007). The overproduction of ROS can damage components of the cell including protein, lipids, and DNA, and disrupt the normal mechanism of cellular signaling. However, the cellular antioxidant defense mechanism directly detoxifies ROS. In a biological system, decreased activities of antioxidant enzymes lead to overproduction of ROS and result in oxidative stress-induced damages (El-Tohami, 2012).

To find out the toxic probability of Chocolate Brown HT on the uterine function we observed the antioxidant enzyme activities in rat models. In this study, we have found that the activities of antioxidant enzymes like SOD, CAT, GPx, GR, and GST were decreased significantly and the amount of malondialdehyde (MDA), a biomarker of lipid peroxidation was increased in a significant way in Chocolate Brown HT exposed rats. The increase in MDA level induces the peroxidation of cellular lipids. So, Chocolate Brown HT impairs the normal physiological mechanisms of the uterus probably by producing oxidative stress in uterine tissues through the inhibition of antioxidant enzymes.

The histological study depicts the significant change in the wall structure of the uterus in Chocolate Brown HT exposed rats compared to control rats. Significant degenerations and lesions were found in the wall structure of the uterus. The resulting necrosis as perceived in the wall structure of the uterus is due to the production of oxidative stress in the uterus.

From the results it may be concluded Chocolate Brown HT impairs the function of the uterus probably by producing oxidative stress-induced damages of uterine tissues (Fig. 4).

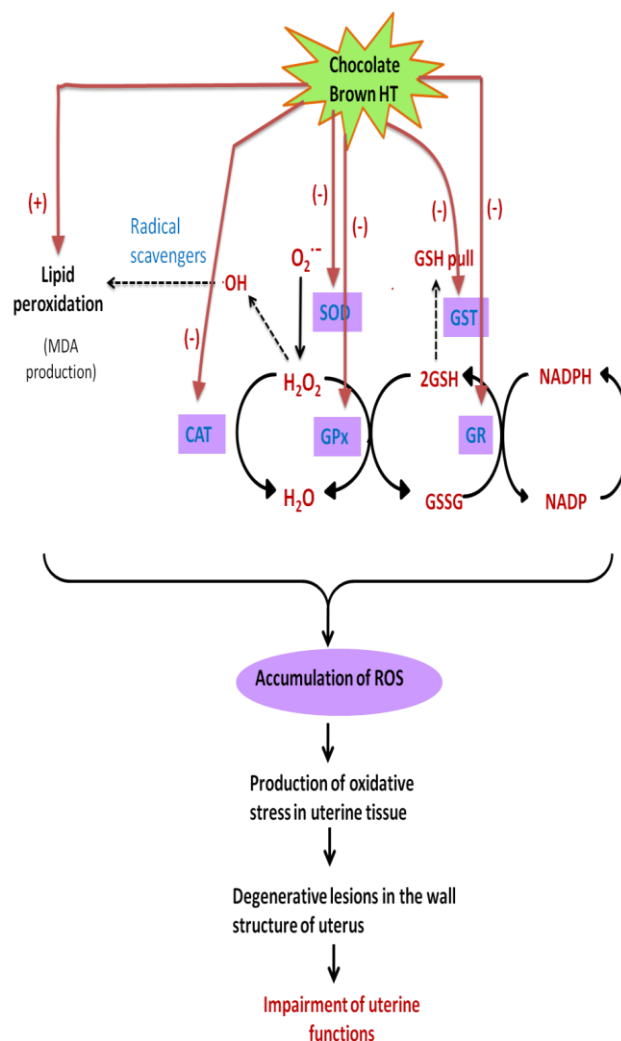


Fig. 4 Schematic representation showing the role of Chocolate Brown HT in inducing oxidative stress-induced damages of uterine tissues. [(+) stimulatory effect, (-) inhibitory effect]

Conclusion

From our study, it may be concluded Chocolate Brown HT impairs the function of the uterus probably by producing oxidative stress-induced damages of uterine tissues.

Acknowledgment

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Conflict of interest

We declare no potential conflicts of interest in this research study and publication of the results of the research.

Ethical Considerations

The study was approved by the ethical committee.

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