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Tartrazine, a male reproductive supressor in adult albino rats

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Abstract: **The present study is aimed to investigate the toxic effects of synthetic azo dye, tartrazine on reproductive toxicity in male adult rats. The rats were administered with tartrazine at doses of 100mg/kg bwt, 300 mg/kg bwt and 500 mg/kg bwt for 60 days. The body weights and weights of reproductive organs decreased in tartrazine administered groups as compared to control group. Results also revealed significant decrease in sperm parameters in tartrazine administered animals over controls. The circulatory level of testosterone was significantly low with increased activity levels of LH and FSH in tartrazine treated rats as compared to controls. Activity levels of the antioxidant enzymes, SOD, CAT, GR and Gpx were significantly reduced with increased activity levels of Lpx in epididymis and testis of tartrazine treated groups. The results of the present study concluded that tartrazine reduces testosterone and induces oxidative stress in testis thereby suppressed reproduction in adult male rats.**

Keywords: **Tartrazine, male reproduction, testosterone, oxidative stress, rat.**

1. INTRODUCTION

Male infertility is one of the serious ongoing problems all over the world. During the past few decades, there is gradual increase in the incidence of testicular cancer and other male reproductive disorders. There are several factors such as genetic factors, life style factors, occupational agents, drugs; environmental factors etc. influence this infertility. Of course among those factors environmental factors have strongly been suspected to play a major role. Many investigators have been strongly suspected that the toxic effects of environmental pollutants are causative agents for male infertility (Sharpe, 1993; Becker and Berhane, 1997; Hirsh, 2003; Brugh and Lipshultz, 2004). However, the other factors also influence the infertility, life style factor is one among, especially food habits, the consumption of readymade foods, junk foods and soft drinks became part of modern life style.

It is well known that addition of food additives is common in the food industries. Colour is one of the important food additives and there is greater usage of food colours is increased to attract people to consume more food. Due to inexpensive and easy availability of synthetic dyes replace the natural colours. These synthetic azo dyes are used extensively in food industry, pharmaceuticals & cosmetics. Azo dyes, one of the most commonly used colouring agents in food processing industry from many years without concern of health effects (Mekkawy *et al.,* 1998; Boussada *et al.,* 2017). Tartrazine is synthetic aromatic water soluble an orange colour azo dye. Tartrazine is widely using dye in the several food preparations like juices, sauces, ice-creams etc. Tartrazine is also used in drugs, cosmetics and pharmaceuticals.

Several studies documented that tartrazine can cause several diseases like angioedema, asthma, urticaria [Ram and Ardern, 2001; Miller, 1982; Babu and Shenolikar, 1995]. It was also reported the genotoxic, mutagenic effects (Sasaki *et al*., 2002; Mpountoukas *et al*., 2010; Oliveira *et al*., 2010) and immunotoxic effects of tartrazine (Koutsogeorgopoulou *et al*., 1998; Guendouz *et al*., 2013). The reports also evidenced that Tartrazine as one of the factors accountable for several health problems such as reproductive toxicity (Goutam *et al*., 2010; Mehedi *et al*., 2009; Himri *et al*., 2022; Viswanathan and Krishnamoorthy, 2012; Boussada *et al*., 2017), hepato toxicty (Collier *et al*., 1983; Khayyat *et al*., 2017), and nephro toxicity (Seesuriyachan *et al*., 2007; Khayyat *et al*., 2017). Tartrazine also increase blood glucose level, creatinine, cholesterol and triglycerides (Himri *et al*., 2011). Several studies also demonstrated that the metabolites which were formed by tartrazine were the causative agents of toxicity, it is due to the release of free radicals after the metabolism

which imbalances the anti oxidant enzymes activity levels and lipid peroxidation levels, stimulates the oxidative stress (Nony *et al*., 1980; Pearce *et al*., 2003; Bansal *et al.*, 2005). There are several investigations revealed the oxidative stress effects of tartrazine on brain, kidney, liver [Bansal *et a*l., 2005; Amin *et al*., 2010; Gao *et al*., 2011] and testis [Viswanathan and Krishnamoorthy, 2012; Boussada *et al*., 2017].

Though there are several animal studies reported the effects of tartrazine on hepatic and nephro toxicity, a few studies reported the effects of tartrazine on reproduction in males. Thus, the present study was aimed to evaluate effect of different sub lethal doses of tartrazine on male reproduction in adult male rats.

2. MATERIALS AND METHODS

Chemicals

Tartrazine and other chemicals used for the work were purchased from Himedia chemicals pvt. Limited, Bangalore, India.

Maintenance and Experimental Model

Healthy 32 male adult albino rats with body weights 200± 10 g were purchased from Sri Venkateswara Enterprises, Bangalore. The rats were maintained in the animal house in polypropylene cages under controlled conditions. On the time of experiment standard rat pellet feed and water *ad libitum* was given to the rats. All experiments were performed as stated in the guidelines and the procedures approved by the Institutional Animal Ethical Committee, Resolution. Number 57/2012/(i)/a/CPCSEA/IAEC/SPMVV/ AMP dt.08-07-2012.

All the rats were randomly divided into four groups with eight rats each. First group served as control and received saline. Second, third and fourth groups treated with tartrazine at doses of 100,300 and 500 mg/kg bwt respectively through gavage for 60 days.

Necropsy and Evaluation of Organ Indices

After completion of the 60 days experimental period the rats were for kept overnight fasting. Prior to sacrificing the rats were given high dose of ether which is used as anesthetic and followed by cervical dislocation the body weights were recorded prior to cervical dislocation. From control and experimental groups blood was collected and centrifuged at 2000g for 15 min, kept overnight at 4^{o}C and separated serum was kept at - 20^{0} C for further hormone analysis. Animals were dissected and tissues like testis and epididymis were isolated and washed with ice cold saline and the weights of the tissues were recorded. By using the following formula the tissue somatic index (TSI) was determined:

$$TSI = \frac{\text{Weight of the tissue}}{\text{Weight of the rat}} \times 100$$

Testicular Daily Sperm production

From control and experimental group rat testis, determination of daily sperm production was studied by Blazak *et al*. (1993) method. Testes were homogenized in 0.01% Triton X-100 in 50 mL ice-cold normal saline (0.9% sodium chloride) solution using a mortar and pestle. After allowing the homogenate to settle for 1 min. and proper mixing of sample, the sperm heads number was counted using four chambers of haemocytometer. The number of sperm produced per gram of testicular tissue per day was calculated and the units of DSP were expressed as millions/gm tissue (Robb *et al*., 1978).

Sperm Count

Total sperm count was determined by Belsey *et al*. (1980) method. Briefly, squeezed the fluid from epidydimis in a petri plate containing 0.5 ml of normal saline at 37ºC and was added to 1 ml of the semen diluting fluid and mixed well. One drop of sperm suspension was taken into haemocytometer chamber and placed it in humid place for 10 minutes to settle the sperms. Total sperm number was enumerated and the sperm count was expressed as millions/ml.

Sperm Motility

The motile sperm percentage (% motility) was determined by the method of Belsey *et al*. (1980). On a clear microscopic slide 0.1 ml of sperm suspension fluid was placed covered with a clean cover slip. The sperm motility of at least 10 fields was recorded under microscope.

Sperm Viability

The sperm viability i.e. ratio of live and dead spermatozoa was determined by the method of Talbot and Chacon (1981). Briefly, 0.2 ml of sperm suspension was added to 1% tryphan blue stain and incubated for 15 minutes at 37°C. After incubation, 0.1 ml of the sperm suspension was loaded in Neubour haemocytometer chamber, cover slip was placed and kept untouched to settle for 60 sec. The viability of sperms were counted and expressed as a percentage of viable sperms.

Hypo-osmotic swelling test (HOS test)

Functional integrity of sperm plasma membrane was evaluated using the hypo-osmotic swelling test which was determined by of Jeyendran *et al*. (1992) method. Briefly, 0.1 ml of sperm sample was added to 1.0 ml of hypo-osmotic solution and incubated at 37ºC for 30 minutes. Sperms with coiled tails were noted and the percent of tail coiled sperm was calculated.

Measurement of serum hormonal levels

The activity levels of LH, testosterone and FSH and in both control and experimental rats were measured by using a commercial kit [Master CLIA (Chemi Luminiscent Immunosorbent assay) vast enabled kit]. Each sample was run in duplicate. The intra- and inter-assay coefficients of variation were performed and found to be less than 10% for these assays. The estimated sensitivity of these hormone assays in this method is about100 pg/ml.

Estimations of Biochemical enzyme assays

10% (w/v) tissue homogenate was prepared by using normal saline and the homogenate was centrifuged for 20 min at 800g, 4° C. Discarded the pellet and the supernatant was used to determine the biochemical assays.

Lipid peroxidation

The lipid peroxidation levels in epididymis and testis were estimated according to Ohkawa *et al*. (1979). Thiobarbuturic acid reactive substances (TBARS) level was estimated by measuring end product malondialdehyde (MDA). Briefly, the selected tissue (15%) w/v was homogenized in equal volumes of trichloroacetic acid, following the addition of 0.37% (w/v) of 2- thiobarbutaric acid and 0.25 N Hcl. After, vortexing the mixture gently, the mixture was subjected to the heat for 15 min. in a boiling water bath. After the heating step, the tubes were taken out from the boiling water bath and cooled on the ice to get the precipitate. After centrifuged the sample at 1000g for 15 min the precipitate was removed and the supernatant was collected for the accumulation of TBARS. The absorbance was measured at 532 nm against the blank with all the reagents except the test sample.

Superoxide dismutase

The levels of superoxide dismutase in epididymis and testis were determined by Marklund and Marklund (1974) method. In short, assay mixture was prepared with 300 µl of enzyme source, 2.4 ml of 50 mM Tris- HCl buffer and 300 µl of 0.2 mM pyrogallol contained 1 mM EDTA (pH 7.6), was thoroughly mixed and measured the absorbance at 420 nm at every 10 s time intervals for 3 min. against the blank which contains all the reagents except pyrogallol. The units were expressed as nmol pyrogallol oxidized/ mg protein/ min.

Catalase

The levels of catalase enzyme in epididymis and testis were determined using Claiborne (1985) method. Briefly the assay mixture contained 10 µl of 19mM hydrogen peroxide, 50 µl of enzyme source and 2.40 mL of 50 mM phosphate buffer (pH 7.0). The absorbance was measured at 240nm against blank which contains 10 µl of 19mM hydrogen peroxide , 2.40 mL of 50 mM phosphate buffer (pH 7.0) reagents except the enzyme source at 10s intervals of time for 3 min. The units were expressed in µmol of hydrogen peroxide consumed/mg preotein/min.

Glutathione peroxidase activity

Glutathione peroxidase assay in testis and epididymis were determined by using Mohandas *et al*. (1984) method. In brief, 100µl of 10mM EDTA, 0.1mL of 10 mM sodium azide, 1.59 ml of 100 mM phosphate buffer (pH 7.6), 0.1 ml glutathione reduced, 0.01 ml of 0.2 mM hydrogen peroxide and 0.1ml of enzyme source were mixed and immediately read at 340 nm against blank at 10 s time intervals for 3min in a spectrophotometer. The units were expressed as nanomoles of NADPH oxidized/min/ mg protein.

Glutathione reductase activity

The activity levels of glutathione reductase (GR) in the testis and epididymis was measured using the method Carlberg and Mannervik. (1985). Briefly, to the reaction mixture, 1.75 ml of 100mM phosphate buffer (pH 7.6), 0.1 ml of 10mM EDTA, 0.05 ml of 20mM Glutathione oxidized and 0.1 ml of 200mM NADPH, 2.1 ml with distilled water, 0.1 ml of enzyme source was added. The absorbance was read at 340 nm against blanks at 10 sec intervals for 3min. The activity of GR was expressed as nanomoles of NADPH reduced/min/ mg protein.

Statistical analysis of data

The mean differences for significance of data were statistically analyzed using Graph pad prism followed by the Tukey test. The data are expressed as mean ± standard deviation. The limit of significance was set at $p < 0.001$

3. RESULTS

During the experimental period mortality rate was not observed in control and experimental groups. The loss of body weight in tartrazine treated groups was observed when compared to control group. Significant ($p<0.001$) decrease in the weights of testis and epididymis was observed in 100,300 and 500 mg/kg bwt tartrazine treated animals when compared to controls (Table 1).

The results of DSP, sperm count, viability, motility, and sperm hypo-osmotic swelling were presented in table 2. The selected sperm parameters were significantly ($p<0.001$) decreased in 100,300 and 500 mg/kg bwt tartazine treated groups over controls. The serum testosterone levels were significantly ($p < 0.001$) decreased in tartrazine administered groups when compared to controls (Fig 1). Simultaneously, the levels of LH and FSH were significantly ($p < 0.001$) increased in tartrazine treated groups over controls (Fig 2).

The levels of malonaldehyde (MDA) were significantly ($p<0.001$) increased in testis and epidydymis of tartrazine administered groups when compared to controls (Table 3 & 4). The levels of super oxide dismutase, catalase, glutathione peroxidase and glutathione reductase were significantly ($p<0.001$) decreased in 100, 300 and 500 mg/kg bwt tartrazine treated animals when compared to controls (Table 3 & 4).

4. DISCUSSION

The present study reveals notable changes caused by oxidative stress induced with the treatment of tartrazine in the epidydimis and testis of rats. In the present study, significant decrease in the body weights observed in tatrazine treated rats compared to control rats. We observed a significant decrease in the weights of testis and epididymis in tartrazine administered groups compared to controls. The results of present work were correlated with previous studies when adult rats exposed to tartrazine, suggesting damage of reproductive organs (Mathur *et al*., 2005; Gautam *et al*., 2010; Himri et al., 2011; Boussada *et al*., 2017). It is well known that the weight of testis depends upon differentiated spermatogenic cells, decreasing weight of testis is indicates the damage of spermatogenesis. Morphology and functional integrity of testis, epididymis and other accessory organs are primarily depends on testosterone. The reduced weight of epididymis and testis in tartrazine administered rats may be decreased spermatogenesis and steroidogenesis.

Sperm analysis is an essential and primary step in the evaluation of fertility index in males. In the present study, a significant decrease in the DSP, sperm count, viability, motility, and HOS- tail coiled sperms observed in tartrazine administered rats. The present results are agreement with previous studies, where administration of tartrazine decreased sperm quality and quantity (Mehedi *et al*., 2009; Gautam *et al*., 2010; Himri *et al*., 2011; Boussada *et al*., 2017). Significant decrease in DSP, sperm count, viability and motility indicating the toxic effects of tartrazine on testes and epididymis of rats. The reduced HOS-tail coiled sperms reveal the damage of membrane integr6ity in sperms of rats by tartrazine.

Testosterone plays an essential role in upholding spermatogenesis, growth and functioning of accessory sex organs. The hormones FSH and LH are accountable for the stimulus of spermatogenesis and steroidogenesis correspondingly. In the present study, decreased serum testosterone levels with increased LH and FSH in tartrazine administered rats. The present results are in agreement with earlier study, Bousaada *et al*. (2017) reported the tartrazine treatment to rats significantly decreased the testosterone levels.

Decline in sperm count might be reduction in weight of epididymis. Decrease in sperm quality and quantity indicates damage of spermatogenesis and steroidogenesis. Because of androgens plays a central role absence or reduction in testosterone indicated decrease in male fertility. Several studies reported that accessory sex organs and testis weights are recognized as constant indices of androgen production from testis (Rind *et al.*, 1963; Price and Williams-Ashman, 1961). Previously it was reported that the decreased testosterone decreases sperm count, motility and viability and spermatogenesis (Gautam *et al*., 2010; Boussada *et al*., 2017).

In the present study, a significant increase in the levels of MDA with decreased levels of antioxidant enzymes SOD, CAT, GPx and GR, observed in testis and epidydimis of rats administered with tartrazine. The elevated levels of MDA after treatment with tartrazine indicate the generation of ROS means it induced oxidative stress in testis and epidydimis. To defense the oxidative stress tissues, testis and epidydimis need to synthesize the antioxidant enzymes. SOD is first antioxidant enzyme which against oxidative stress and converts superoxide anions to hydrogen peroxide in dismutation. Catalase is other enzyme which neutralizes the hydrogen peroxide to molecular oxygen and water. Glutathione related enzymes, glutathione reductase and glutathione peroxidase also act against oxidative damage. GPx metabolizes hydrogen peroxide to protect cell membranes from lipid peroxidation and GR is responsible for suporting the intracellular concentration of reduced glutathione. But in the present study, a significant decrease in the levels of the anti oxidant enzymes in testes and epidydimis of rats treated with tartrazine indicates production of free radicals induce damage of antioxidant defense system in testis (Viswanathan and Krishna moorthy, 2012). It was already reported that, Tartrazine metabolizes into aromatic amine by the bacteria in the intestine and generates the ROS (Moutinho *et al*., 2007). As a consequence of the formation of the ROS the oxidative damage induced and a significant increase in the lipid peroxidation (Bansal, 2005; Viswanathan and Krishna moorthy, 2012).The present results were also consistent with earlier reports (Viswanathan and Krishna moorthy, 2012; Boussada *et al*., 2017). Besides in testis, it was demonstrated that administration tartrazine decreased antioxidant levels with elevated levels of LPx in liver and kidney of rat and mice (Collier *et al.*, 1983; Seesuriyachan *et al.*, 2007; Amin et al., 2010; Omca *et al*., 2012; Khayyat *et al*., 2017).

5. CONCLUSION

Sperm failure is became common problem in 3-5% people in the world. Addressing this problem and route cause in humans is complicated and finding ways are in pipeline. The present study in the series and reveals the administration of tartrazine induces oxidative stress, decreases sperm production, sperm quality and quantity in rats. The data of the present study may extrapolate to humans and can prevent the tartrazine induced suppression of male reproduction.

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APPENDICES - A

List of Table:

Table 1: Tartrazine induced body weight (g) and organ weight changes in rats:

Parameter	Control	Tartrazine (100 mg/kg bwt)	Tartrazine (300 mg/kg bwt)	Tartrazine (500 mg/kg bwt)
Body weight	310.5±10.12	286.4 [a] ±12.64 (-7.76)	280.5 [b] ±14.52 (-9.66)	271.8 [c] ±11.65 (-12.46)
Testis	1.10±0.05	1.02 [a] ±0.03 (-7.27)	0.95 [b] ±0.02 (-13.63)	0.76 [c] ±0.035 (-30.90)
Epididymis	0.78±0.02	0.74 [a] ±0.02 (-5.12)	0.71 [b] ±0.02 (-8.97)	0.68 [c] ±0.03 (-12.82)

Values are mean ± S.D. of 8 individuals for each treatment group. Values in parentheses are percent change from that of the control. Mean values with different superscripts in a row differ significantly from each other at $p < 0.001$.

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Table 2: Effect of Tartazine on selected sperm parameters in adult rats

Parameter	Control	Tartrazine(100 mg/kg bwt)	Tartrazine 300 mg/kg. b.wt	Tartrazine 500 mg/kg b.wt
Daily sperm production (millions/g testes)	22.15±1.78	21.16 [a] ±1.64 (-44.69)	19.24 [b] ±1.52 (-13.13)	17.56 [c] ±1.34 (-20.72)
Sperm count (millions/mL)	75.16±7.02	69.18 [a] ±7.01 (-7.95)	62.14 [b] ±6.92 (-17.32)	59.61 [c] ±5.84 (-20.68)
Motile sperm (%)	77.36±4.61	72.38 [a] ±4.36 (-6.43)	69.21 [b] ±4.95 (-10.53)	66.86 [c] ±4.95 (-13.57)
Viable sperm (%)	76.54±5.68	74.92 [a] ±5.16 (-2.11)	69.27 [b] ±4.92 (-9.49)	65.34 [c] ±4.96 (-14.63)
HOS- tail coiled sperm (%)	65.21±3.95	62.67 [a] ±2.96 (-3.89)	60.84 [b] ±2.98 (-6.70)	59.54 [c] ±2.61 (-8.69)

Values are mean ± S.D. of 8 individuals for each treatment group.

Values in parentheses are percent change from that of the control.

Mean values with different superscripts in a row differ significantly from each other at p<0.001.

List of Figure:

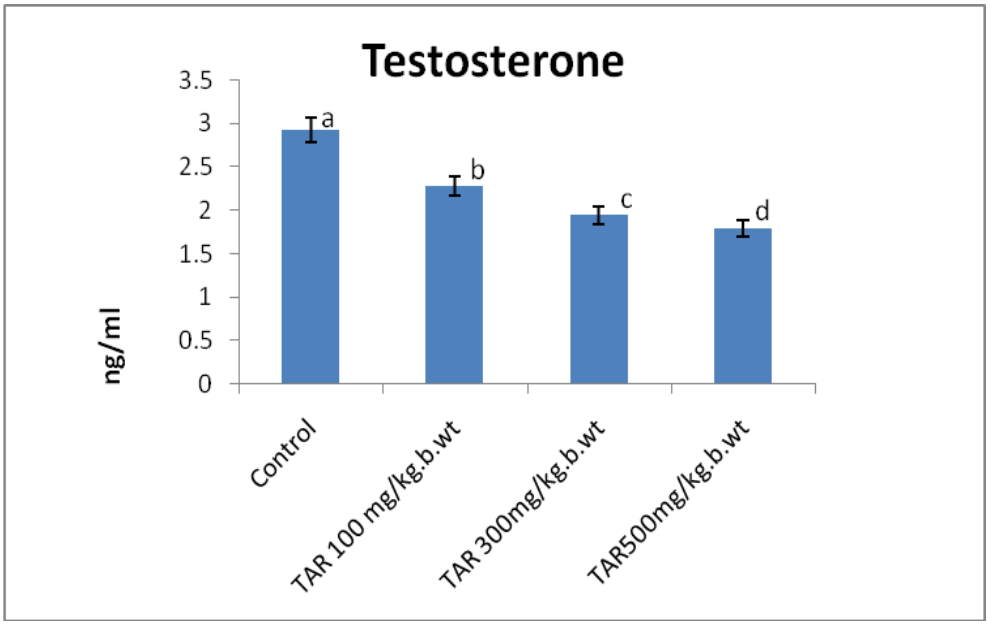

Fig 1: Effect of Tartazine on serum testosterone levels in adult rats

Testosterone levels in testis of control and tartrazine treated with two doses.

The column bars are mean ± SD of 8 individuals. Bars denoted with different superscripts are significantly different from different groups ($p < 0.001$)

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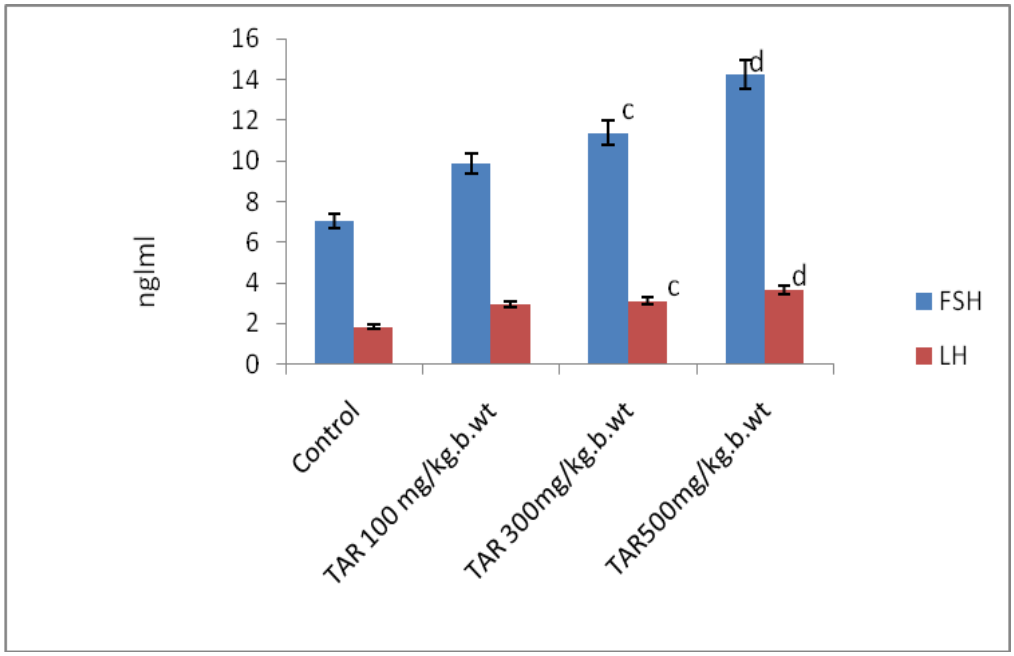

Fig 2: Effect of Tartazine on serum FSH, and LH activity levels in adult rats.

FSH, LH levels in testis of control and tartrazine treated with two doses. The column bars are mean ± SD of 8 individuals. Bars denoted with different superscripts are significantly different from different groups ($p < 0.001$)

Table 3: Effect of Tartrazine on anti-oxidant enzyme levels in Testis tissue of adult rats

Parameter	Control	Tartrazine (100 mg/kg bwt)	Tartrazine (300 mg/kg bwt)	Tartrazine (500 mg/kg bwt)
Lipid peroxidation(µ molmalondialdehyde per g tissue)	11.68±1.09	13.62 [a] ±1.11 (16.60)	15.68 [b] ±1.12 (34.24)	17.85 [c] ±1.09 (52.82)
Super oxide dismutase(nmolpyrogallol oxidized per mg protein per min)	7.15±0.62	6.39 [a] ±0.53 (-10.62)	5.34 [b] ±0.05 (-25.31)	4.25 [c] ±0.13 (-40.55)
Catalase(nmol H_2O_2 metabolized per mg protein per min)	16.52±1.82	14.95 [a] ±1.19 (-9.50)	14.12 [b] ±1.16 (-14.52)	13.26 [c] ±1.54 (-19.73)
Glutathione peroxidase (nmol NADPH oxidized per mg protein per min)	42.73±1.97	35.62 [a] ±2.92 (-16.63)	34.25 [b] ±2.14 (-19.84)	29.65 [c] ±1.88 (-30.61)
Glutathione reductase (nmol NADPH oxidized per mg protein per min)	47.26±4.32	44.38 [a] ±3.94 (-6.09)	40.65 [b] ±3.41 (-13.98)	34.15 [c] ±3.51 (-27.74)

Values are mean ± S.D. of 8individuals for each treatment group.

Values in parentheses are percent change from that of the control.

Mean values with different superscripts in a row differ significantly from each other at

$p<0.001$

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Table 4: Effect of Tartrazine on anti- oxidant enzyme levels in epididymis tissue of adult rats

Parameter	Control	Tartrazine (100 mg/kg bwt)	Tartrazine (300 mg/kg bwt)	Tartrazine (500 mg/kg bwt)
Lipid peroxidation(μ molmalondialdehyde per g tissue)	7.09±0.39	9.71 [a] ±0.81 (36.95)	11.71 [b] ±1.09 (65.16)	14.21 [c] ±1.72 (100.42)
Super oxide dismutase(nmolpyrogallol oxidized per mg protein per min)	5.07±0.52	3.94 [a] ±0.41 (-22.28)	2.03 [b] ±0.49 (-59.96)	1.98 [c] ±0.27 (-60.94)
Catalase(nmol H_2O_2 metabolized per mg protein per min)	8.32±0.69	4.35 [a] ±0.91 (-47.71)	4.16 [b] ±0.56 (-50.00)	3.64 [c] ±0.42 (-56.25)
Glutathione peroxidase (nmol NADPH oxidized per mg protein per min)	23.47±4.31	17.14 [a] ±3.81 (-26.97)	15.91 [b] ±3.71 (-32.21)	13.46 [c] ±2.14 (-42.65)
Glutathione reductase (nmol NADPH oxidized per mg protein per min)	26.67±1.49	20.82 [a] ±1.34 (-21.93)	14.09 [b] ±1.22 (-47.25)	12.23 [c] ±1.13 (-54.14)

Values are mean ± S.D. of 8individuals for each treatment group.

Values in parentheses are percent change from that of the control.

Mean values with different superscripts in a row differ significantly from each other at

$p<0.001$