



## $\alpha$ -lipoic acid inhibits oxidative stress in testis and attenuates testicular toxicity in rats exposed to carbimazole during embryonic period



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### ABSTRACT

The aim of this study was to evaluate the probable protective effect of  $\alpha$ -lipoic acid against testicular toxicity in rats exposed to carbimazole during the embryonic period. Time-mated pregnant rats were exposed to carbimazole from the embryonic days 9–21. After completion of the gestation period, all the rats were allowed to deliver pups and weaned. At postnatal day 100, F1 male pups were assessed for the selected reproductive endpoints. Gestational exposure to carbimazole decreased the reproductive organ indices, testicular daily sperm count, epididymal sperm variables *viz.*, sperm count, viable sperm, motile sperm and HOS-tail coiled sperms. Significant decrease in the activity levels of 3 $\beta$ - and 17 $\beta$ -hydroxysteroid dehydrogenases and expression of StAR mRNA levels with a significant increase in the total cholesterol levels were observed in the testis of experimental rats over the controls. These events were also accompanied by a significant reduction in the serum testosterone levels in CBZ exposed rats, indicating reduced steroidogenesis. In addition, the deterioration of the testicular architecture and reduced fertility ability were noticed in the carbimazole exposed rats. Significant reduction in the activity levels of superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and reduced glutathione content with a significant increase in the levels of lipid peroxidation were observed in the testis of carbimazole exposed rats over the controls. Conversely, supplementation of  $\alpha$ -lipoic acid (70 mg/Kg body-weight) ameliorated the male reproductive health in rats exposed to carbimazole during the embryonic period as evidenced by enhanced reproductive organ weights, selected sperm variables, testicular steroidogenesis, and testicular enzymatic and non-enzymatic antioxidants. To conclude, diminished testicular antioxidant balance associated with reduced spermatogenesis and steroidogenesis might be responsible for the suppressed reproduction in rats exposed to the carbimazole transplacentally. On the other hand,  $\alpha$ -lipoic acid through its antioxidant and steroidogenic properties mitigated testicular toxicity which eventually restored the male reproductive health of carbimazole-exposed rats.

### 1. Introduction

Antithyroid drugs continue to be the first-line treatment to manage hyperthyroidism and also to control thyroid function before surgery. The mode of action of antithyroid drugs is via blockage of iodine organification and iodine-tyrosine coupling, thereby biosynthesis of thyroid hormones (THs) from the thyroid gland. Antithyroid drugs such as polypropylthiouracil (PTU), carbimazole (CBZ) and its metabolite methimazole (MMI) are widely used all over the world [1]. Despite their promising therapeutic potential, the clinical success of antithyroid drugs is often limited by deleterious side effects such as nephrotoxicity,

neurotoxicity, hepatotoxicity, acute pancreatitis, thyroid carcinoma and testicular toxicity [2–6]. Further, there is a major concern towards the usage of antithyroid drugs in pregnant women, because these drugs can readily cross the placenta and affect fetal development [1]. Rodent studies indicated that perinatal exposure to CBZ causes disorganization of thyroid gland [7] and administration of PTU and MMI during the pre- (via the placenta) and peri-natal (via the placenta and through the milk) periods not only resulted in neurotoxicity [8] but also caused testicular and epididymal toxicity accompanied by the reduced serum testosterone levels [9–13]. Further, studies of Calanis-Continente et al. [4] reported the clinical history of a woman with a necrotizing

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glomerulonephritis and pulmonary hemorrhage associated with CBZ therapy.

Interestingly, perinatal exposure to PTU and/or neonatal exposure to MMI resulted in oxidative stress characterized by the reduced antioxidant capacity as well as the disrupted architecture of testis tissue in rats [14–16]. In addition, studies of Sakr et al. [6] demonstrated that oxidative stress and reactive oxygen species play an important role in the pathogenesis of CBZ-induced testicular toxicity in adult rats. Thus, it seems apparent that, the mechanism(s) of reproductive toxicity caused by antithyroid drugs is based on the suppression of steroidogenesis or the generation of oxygen free radicals and/or both.

$\alpha$ -lipoic acid (LA; 1,2, dithiolane-3-pentanoic acid) is an essential cofactor for several enzymes that mediate energy production (acetyl-CoA synthesis from pyruvic acid), fatty acid metabolism (acyl CoA synthesis), TCA cycle ( $\alpha$ -ketoglutaric acid to succinyl-CoA) and nucleotide synthesis (glycine synthesis) [17]. It also exhibits extraordinary antioxidant properties [18]. Moreover, because of its lipophilic nature, it can easily cross cell membranes and along with its redox couple, dihydrolipoic acid (DHLLA) quenches the free radicals more efficiently in both lipid and aqueous compartments [19]. Previously, the protective effects of LA on the testicular steroidogenesis in male fetuses of dams exposed to dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been reported [20]. Studies of Dixit et al. [21] reported that the administration of LA on postnatal days (PND) 4–15 triggered the enzymatic and non-enzymatic antioxidants, superoxide dismutase and reduced glutathione (GSH), respectively in the hypothalamus of arsenic exposed rats. *In vitro* studies also indicated that LA with its antioxidant properties, protect the oocytes and the embryos retrieved from the mice injected with nicotine [22]. Furthermore, endogenous lipoic acid metabolism during the fetal period of mice has been demonstrated [23]. These findings highlighted the importance of LA during the critical phases of the development. With respect to male reproduction, a significant number of therapeutic studies suggested that LA protects the testis and its functions against a broad spectrum of toxic insults where oxidative stress is a part of the underlying etiology [24,20,25,26]. Studies of Jung et al. [27] reported that LA protects the thyroid gland from radiation-induced oxidative damage in the rats. In addition, anticancer and anti-apoptotic [28] properties of the LA has also been reported. Human studies indicated that the antioxidant LA showed amelioration of endothelial functions against subclinical hypothyroidism-induced oxidative stress [29].

The present study was aimed to investigate whether the administration of carbimazole during the embryonic period affects the testicular functions in adulthood and if so, whether the supplementation of LA protects the testis and its functions in adult offspring rats.

## 2. Materials and methods

### 2.1. Chemicals

Alpha lipoic acid was obtained from the Sigma chemicals Co. (St. Louis, MO, USA). Carbimazole which was available under the trade name Neo-mercazole (Abbott Healthcare Pvt. Ltd., Himachal Pradesh, India (Batch No: NZB6008) was purchased from a local pharma drug store, Nellore. All other chemicals and kits used in the present study were of reagent grade and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA), and Merck Laboratories (India).

### 2.2. Animals

Healthy male ( $n = 10$ ) and female ( $n = 10$ ) albino rats of Wistar strain were obtained from the Narayana Medical College, Nellore, India. Male and female rats were housed ( $n = 4$  per cage) separately in standard polypropylene cages ( $18'' \times 10'' \times 8''$ ) containing the sterile paddy husk as the bedding material. All the animals were acclimatized over a period of 2 weeks under the controlled laboratory conditions

[Temperature: 22–25 °C; light: 12 h light/dark cycle and the relative humidity:  $50 \pm 5$  °C]. The rats had free access to standard chow (M/S Hindustan Animal Feeds, India) and water. All the experiments performed in this study were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India [30] and approved by the Institutional Animal Ethical Committee (vide No. 1558/PO/a/11/CPCSEA/RIP/2015/03-10). After completion of the acclimatization period, virgin female rats with proestrus ( $160 \pm 10$  g) were cohabited with the fertile male rats (one male and one female). Copulation was examined by the presence of a sperm and/or vaginal plug in a vaginal smear. The cohabitation period was five days. The presence of vaginal plug and/or sperm in a vaginal smear was considered as the day 1 of pregnancy or gestation day 1 (GD 1) and accordingly, on the day when copulation was confirmed, pregnant rats were separated from males and transferred to the new cages (one per cage) to start the experiment.

### 2.3. Experimental design

Time-mated pregnant rats (GD 1) were randomly assigned to control or carbimazole groups. Rats in the control group ( $n = 10$ ) did not receive any treatment, whereas rats in the carbimazole group were orally administered with the antithyroid drug, carbimazole via drinking water at a dose of 1.35 mg/Kg body weight from days 9–21 of pregnancy. The time line, GD 9–21 was selected because the fetal differentiation of vital organs, including the testis occurs during this critical period [11]. The dosage of the carbimazole was selected from previous studies (6). Moreover, the dosage of the carbimazole used in this study was equivalent to the therapeutic dose prescribed to the humans [31]. The selection of carbimazole was based on our discussion with Dr. T. Ramalinga Reddy, Thyroidologist, Sarayu Hospitals, Nellore, AP, India.

The rats were allowed to deliver the pups. The F1 generation male rats were weaned and injected intraperitoneally (i.p) with either  $\alpha$ -lipoic acid (70 mg/Kg bodyweight) or vehicle (20  $\mu$ l) on PND 3–14 covering the critical development of testis [32] and the brain [28]. The dosage of the lipoic acid (LA) was based on the studies of Dixit et al. [28]. On PND 100, male rats from control, transplacental CBZ-exposed and CBZ-exposed lipoic acid injected groups were housed as breeding pairs with normal cycling females (90 days old) to be parents of the F2 generation. The mating period was 5 days and when copulation was confirmed or the 5-day-mating period was completed, the F1 generation male rats were fasted overnight, weighed, and killed by cervical dislocation. Animals were humanely sacrificed and the liver, kidney, brain, testes, epididymis, seminal vesicles, vas deferens and ventral prostate were collected immediately, weighed to the nearest milligram using an Electric balance (Shimadzu Model: BI-220H). Tissue somatic index (TSI) was determined using the following formula: [(weight of the tissue/weight of the rat)  $\times$  100].

During the weaning period, physical developmental landmarks such as the bodyweight, eye-opening, fur appearance, pinna-unfolding, incisor-emergence day were evaluated from PND 2 to PND 21.

### 2.4. Fertility examinations

Control and carbimazole-exposed rats injected with or without  $\alpha$ -lipoic acid were cohabited with the normal cycling females (90 days old) of proven fertility. The cohabitation period was restricted to 5 days. Every morning, vaginal smears were collected and examined microscopically for the presence of sperm. The presence of sperm in the vaginal smear was considered as the day 1 of gestation. The number of successful mating was calculated on the presence of sperm in the vaginal plug in the morning. The mating- and fertility-indices were determined by the formula: [(number of sperm positive females/number of pairing)  $\times$  100] and [(number of pregnant rats/number of sperm positive females)  $\times$  100], respectively. Total number of pups delivered to respective females cohabited with the control and experimental rats

were recorded. The weights of pups were also recorded.

### 2.5. Semen analysis

The cauda part of epididymis was isolated at autopsy and chopped in a Petri dish (10 ml) containing 2 ml of physiological saline (0.9% NaCl) at 37 °C to get a sperm suspension. Sperm parameters such as sperm count and progressive sperm motility were performed according to Belsey et al. [33]. The viability of the sperm was determined using 1% trypan blue reagent [34]. The sperm membrane integrity was determined by exposing the sperms to hypo-osmotic solution and observed for coiled tails under the microscope [35]. The motility, viability and HOS-coiled sperms were expressed as a percentage of total sperm counted. The sperm count was expressed as millions/ml. Daily sperm production (DSP) from the testis was performed according to the method described by Blazak et al. [36]. The units of DSP were expressed as millions/g testis. Morphological changes in the sperms were identified according to the protocol described by Linder et al. [37]. One hundred sperms from each animal were evaluated and analyzed for the sperm abnormalities such as head defect, middle piece defect, tail defect and detached head. The morphological changes in the sperms were determined by light microscope (Model no. HV-12TR) and the percent of abnormality was calculated.

### 2.6. Testicular cholesterol levels

Total cholesterol levels in the testis of rats were determined by using the method of Zlatkis et al. [38]. Briefly, 0.2 ml of testicular homogenate prepared in 5 ml of ferric chloride solution (0.05% of FeCl<sub>3</sub> 6H<sub>2</sub>O in glacial acetic acid) was added to a test tube. After thorough mixing, 3 ml of sulphuric acid was added and the test tubes were then allowed to stand for 20 min. The incubation mixture deprived of testicular homogenate was used as the blank. The absorbance was recorded at 540 nm on a spectrophotometer. The cholesterol levels were expressed as mg/g tissue weight.

### 2.7. Testicular steroidogenic marker enzyme activity levels

The activity levels of testicular steroidogenic marker enzymes: 3β hydroxysteroid dehydrogenase (3β HSD) (EC 1.1.1.51) and 17β hydroxysteroid dehydrogenase (17β HSD) (EC 1.1.1.64) was determined as per the protocol described by Bergmeyer [39]. Briefly, testis was homogenized (5% w/v) in ice cold Tris-HCl at pH 6.8. The homogenate was centrifuged to separate microsomal fraction which was used as an enzyme source. The reaction mixture (2 ml) contains 0.08 μmol of steroid substrate (dehydroepiandrosterone for 3β HSD and androstenedione for 17β HSD), 100 μmol of cofactors (NADPH for 17β HSD and NAD for 3β HSD) and 0.1 M pyrophosphate buffer (pH 7.4) and 20 mg of enzyme source. The incubation mixture deprived of substrate(s) was used as reagent blank. The absorbance was recorded at 340 nm on a spectrophotometer. The units for 3β – and 17β – HSDs were expressed as nmol of NAD converted to NADH/mg protein/min and nmol of NADPH converted to NADP/mg protein/min, respectively.

### 2.8. Antioxidant enzyme assays

Determination of testicular lipid peroxidation and enzymatic antioxidants were performed as per the method described by Kaushal et al. [40]. Briefly, testis (10% W/V) was thoroughly homogenized in isolation medium [ice-cold sucrose (0.25 M), Tris-HCl (10 mM), EDTA (1 mM) and BSA (250 μg/ml); pH 7.4] with the help of a motor-driven glass Teflon homogenizer. The homogenate was centrifuged at 1000g for 10 min at 4 °C to separate the cell debris and nuclei. The obtained supernatant was used for enzymatic evaluations and TBARS content. Aliquots of the extracts were examined separately for each enzyme. Superoxide dismutase (SOD) activity was determined based on its

ability to inhibit the autooxidation of epinephrine at alkaline medium at 480 nm (Spectrophotometer Make: Analytical Technologies; Model No: 2060) [41]. The activity of catalase (CAT) was determined by its ability to decompose hydrogen peroxide (10 mM solution) concentration at 240 nm [42]. The activity levels of glutathione peroxidase (GPx) were determined through the system glutathione/NADPH glutathione reductase by the dismutation of *tert*-butylhydroperoxide at 340 nm [43]. Activity of glutathione reductase (GR) was determined through oxidation rate of NADPH in a reaction medium containing 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (pH 7.0) containing 0.1% DPTA and 0.1 mM oxidized glutathione (GSSG) in a buffer [44].

### 2.9. Glutathione (GSH) assay

GSH was measured according to Beutler [45], using the Elman's reagent (DTNB). Briefly, after tissue excision, the tissue acid extracts were obtained immediately by the addition of 12% trichloroacetic acid. (1:4 v/v) and then centrifuged. The resulting supernatant was added to 0.25 mM DTNB in 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (pH 8.0) and the formation of thiolate anion was measured at 412 nm.

### 2.10. Lipid peroxidation (LPx) levels

Determination of LPx in the testicular homogenate was in accordance with the protocol of Ohkawa et al. [46]. The extent of LPx was quantified as the concentration of thiobarbituric acid-reactive product malondialdehyde (MDA). To the 0.5 ml of fresh homogenates prepared in phosphate buffer (pH 7.2), 1.0 ml of trichloroacetic acid and 1.0 ml of TBA were added and mixed thoroughly. The mixture was heated in a boiling water bath for 20 min, cooled and the tubes were centrifuged at 1000g for 10 min. The absorbance was then measured spectrophotometrically at 535 nm. The MDA equivalents of the samples were calculated using the extinction coefficient  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### 2.11. Estimation of proteins

The protein content in the testis was determined as per the method Lowry et al. [47] using bovine serum albumin as a standard.

### 2.12. Testicular StAR mRNA levels (RT-PCR)

Total RNA was extracted from the testis by using Trizol plus RNA purification system (Invitrogen). After extraction, the purity and concentrations of RNA were analyzed by spectroscopic analysis by recording OD values at 260 and 280 nm (OD 260/OD 280 = > 1.8–2.0) and by agarose gel electrophoresis. Complementary cDNA was synthesized from total RNA (1 μg) by using iscript™ cDNA synthesis kit (Biorad, India) as described in the manufacturer's protocol. Semi-quantitative PCR (Applied Biosystems, SimpliAmp™, Thermal cycler) was performed to determine the expression levels of StAR and GAPDH (internal control). The PCR reaction mixture contains 10 μl of Phusion mixture (Thermo Scientific), 1 μl of forward primer (FP), 1 μl of reverse primer (RP), 2 μl of cDNA and 6 μl of nuclease free water and the PCR parameters consisted of 3 steps where step 1 includes 1 cycle of 95 °C, for 30 s followed by 30 cycles of 95 °C for 5 s, 55 °C for 15 s (step 2). Final step includes 1 cycle of 72 °C for 10 min. The following sets of forward and reverse primers were used for the present study to amplify StAR [Gene bank accession number: NM031558; FP: 5-TTGGGCATACTCAACAACCA-3' (nucleotide region: 375–394) and RP: 5-ATGACACCGCTTTGCTCAG-3' (nucleotide region: 745–763)] and GAPDH [Gene bank accession number: NM017008; FP: 5-AGACAGCCGCATCTTCTTGT-3' (nucleotide region: 28–47); R: 5-CTTGCCGTGGGTAGAGTCAT-3' (nucleotide region: 215–234)] from the testis of controls and experimental rats. The PCR products were run on 1.8% agarose gels in TAE buffer and the relative intensities of the StAR bands were normalized against corresponding GAPDH band

**Table 1**  
Effect of injection of lipoic acid (LA) on *in vivo* fertility assays of rats exposed to carbimazole (CBZ) during embryonic period.

Parameter	Groups			
	Normal	LA-supplemented	CBZ-exposed	CBZ-exposed + LA supplemented
Number of litters	12	12	12	12
No. of mating trails (days)	1.4 <sup>a</sup> ± 0.38	1.61 <sup>a</sup> ± 0.32	4.42 <sup>b</sup> ± 0.52	2.12 <sup>c</sup> ± 0.42
Mating Index (%)	100 (12/12)	100 (12/12)	66 (8/12)	83 (10/12)
Fertility Index (%)	100 (12/12)	100 (12/12)	100 (8/8)	100 (10/10)
No. of live fetuses/rat	12.31 <sup>a</sup> ± 0.45	11.68 <sup>a</sup> ± 0.53	6.24 <sup>b</sup> ± 0.68	9.64 <sup>c</sup> ± 0.61
Body weight of pups (g)	5.62 <sup>a</sup> ± 1.62	5.54 <sup>a</sup> ± 1.26	5.34 <sup>a</sup> ± 1.79	5.29 <sup>a</sup> ± 1.22

Data are expressed as mean ± S.D. <sup>a-c</sup> Different superscripts in the same row indicate a significant difference ( $p < 0.05$ ).

intensities.

### 2.13. Serum testosterone levels

Prior to necropsy, blood was collected from the heart using a heparinized syringe from each animal. The serum was separated from the blood by centrifugation at 2000g for 15 min after overnight storage at 4 °C and stored at –20 °C until all of the samples were collected. Serum testosterone levels of the control and experimental F1 rats were analyzed by using an enzyme linked immunosorbent assay (ELISA) kit [Diametra, Italy] purchased from the local suppliers. The assay was done strictly as per the protocol mentioned in the kit. All the samples were run at the same time to avoid interassay variation. The sensitivity of the test ranges between 0–16 ng/ml. The serum level of testosterone was expressed as ng/ml.

### 2.14. Histology of testis

Testis from each rat of control and experimental groups was individually fixed in the Bouin's solution for 24 h, dehydrated with ascending alcoholic series, cleaned in xylol and embedded in the paraffin wax. The sectioned specimens were stained with hemotoxylin and eosin Y for histological studies. The histological findings of the testis were determined using Hovers microscope (Model no. HV-12TR) [48].

### 2.15. In silico studies

The structure of rat StAR protein (UniProt sequence Id: P97826) was modelled using human StAR protein as a template (PDB: 3P0L). The details were given in supplementary material. Previously, it has been shown that the residues Glu169, Arg188, Leu199 and His220 in human StAR protein (3P0L) bind cholesterol, an endogenous ligand [49]. The same residues were 100% conserved in modelled structure of rat StAR protein and was visualized using PyMOL (Supplementary material). Carbimazole and methimazole were also docked against human and rat proteins and were visualized by using Discovery Studio 2.1.

### 2.16. Statistical analysis

Results are reported as mean ± S.D. Statistical analysis was performed using one-way analysis of variance (ANOVA) using post-hoc Tukey test. All statistical tests were performed by using Statistical Package for Social Sciences version 16.0 (SPSS Inc, Chertsey, UK). The differences in the values between the groups were considered significant at  $p < 0.05$ .

## 3. Results

None of the animals was excluded from the experiment and no mortality was observed in any of the control and experimental groups.

The analysis of developmental landmarks such as pinna unfolding, eye opening, anogenital distance, and hair development did not differ

significantly among the F1 male pups delivered to rats in the control and CBZ groups. No mortality was observed in the F1 male pups injected with LA from PND 3–14 in any of the control and experimental groups.

### 3.1. Fertility analysis

CBZ exposed rats showed a significant increase in the number of mating trials to impregnate females as compared to controls. However, a significant reduction in the mating trials was observed in lipoic acid supplemented CBZ exposed rats to impregnate females as compared to CBZ exposed rats. No significant changes were observed in the fertility index between control and experimental groups. A significant ( $p < 0.01$ ) reduction (~49.3%) was observed in the number of pups delivered to mothers cohabited with CBZ exposed rats as compared to controls. Whereas, a significant ( $p < 0.01$ ) increase (~54%) in the number of pups delivered to female rat mated with lipoic acid injected CBZ exposed rats was observed over CBZ exposed rats (Table 1).

### 3.2. Body and organ weight gain profile

No significant changes were observed in the body weights of controls and CBZ exposed rats. However, a significant ( $p < 0.001$ ) reduction in the testicular (30.7%) and accessory sex organ indices [epididymis (23%), vas deferens (34.14%), prostate gland (30.57%) and seminal vesicle (19.05%)] were observed in rats exposed to CBZ as compared to controls. Whereas, a significant increase in the weights of reproductive organs was observed in CBZ exposed rats supplemented with lipoic acid as compared to CBZ exposed rats. On the other hand, no significant differences were observed in the weights of reproductive organs in control rats injected with or without lipoic acid injection (Table 2). Fig. 1 depicts the macroscopic appearance of testis from controls, rats exposed to CBZ *in utero*, and CBZ exposed rats injected with the lipoic acid. There was a size reduction in the testis of rats exposed to CBZ *in utero* as compared to controls, whereas injection of lipoic acid restored the size of the testis in CBZ-exposed rats as compared to CBZ exposed rats. No difference in the weights of liver, brain, and kidney was observed in any of the groups (Table 2).

### 3.3. Sperm quality and quantity

The average daily sperm count in the testis of controls with or without lipoic acid supplementation was found to be  $13.56 \pm 3.14$  and  $12.47 \pm 3.14$  (million/g testis), respectively in rats, whereas testicular daily sperm count was significantly reduced to 33.99% in CBZ exposed rats as compared to controls. Lipoic acid injection restored the daily sperm count in the testis of CBZ exposed rats to 31.4% over CBZ exposed rats (Table 3). Significant depletion in the epididymal sperm count (38.38%), motile sperm (36.28%), viable sperm (39.34%) and coiled sperm (37.17%) was observed in rats exposed to CBZ *in utero*. All the sperms were apparently normal in control rats, whereas many morphological altered sperm (head defects; Fig. 2) were observed in

**Table 2**  
Effect of lipoic acid (LA) on body weights (g) and tissue somatic indices (w/w%) in carbimazole (CBZ)-exposed prenatates at their adulthood.

Tissue	Groups			
	Control <sup>s</sup>		CBZ-exposed*	
	Untreated	LA-treated	Untreated	LA-treated
Body Weight	191.26 <sup>a</sup> ± 10.68	188.38 <sup>a</sup> ± 10.82	179.48 <sup>a</sup> ± 12.71	180.42 <sup>a</sup> ± 6.77
Testes	1.14 <sup>a</sup> ± 0.056	1.08 <sup>a</sup> ± 0.083	0.79 <sup>b</sup> ± 0.104	0.98 <sup>c</sup> ± 0.043
Epididymes	1.008 <sup>a</sup> ± 0.062	0.987 <sup>a</sup> ± 0.126	0.776 <sup>b</sup> ± 0.032	0.868 <sup>c</sup> ± 0.042
Vas deference	0.164 <sup>a</sup> ± 0.0213	0.159 <sup>a</sup> ± 0.0168	0.108 <sup>b</sup> ± 0.0315	0.146 <sup>a</sup> ± 0.0252
Prostate gland	0.157 <sup>a</sup> ± 0.016	0.164 <sup>a</sup> ± 0.029	0.109 <sup>b</sup> ± 0.021	0.143 <sup>a</sup> ± 0.036
Seminal vesicle	0.467 <sup>a</sup> ± 0.063	0.471 <sup>a</sup> ± 0.097	0.378 <sup>b</sup> ± 0.071	0.414 <sup>b</sup> ± 0.036
Brain	0.642 <sup>a</sup> ± 0.045	0.661 <sup>a</sup> ± 0.061	0.639 <sup>a</sup> ± 0.056	0.652 <sup>a</sup> ± 0.081
Liver	3.112 <sup>a</sup> ± 0.262	3.209 <sup>a</sup> ± 0.41	3.105 <sup>a</sup> ± 0.512	2.989 <sup>a</sup> ± 0.37
Kidney	0.678 <sup>a</sup> ± 0.027	0.684 <sup>a</sup> ± 0.057	0.662 <sup>a</sup> ± 0.036	0.654 <sup>a</sup> ± 0.06

Data are expressed as mean ± S.D. of 10 individuals.  
Comprise rats delivered to: <sup>s</sup>unexposed dams and \*dams exposed to CBZ during pregnancy.  
<sup>a-c</sup> Different superscripts in the same row indicate a significant difference (p < 0.05).



**Fig. 1.** Macroscopic pictures of the testes in control (A), CBZ-exposed (B) and LA treated CBZ-exposed (C) rats.

rats exposed to CBZ during the embryonic period. Substitution of lipoic acid to CBZ exposed rats showed recovery of selected epididymal sperm parameters to control levels (Table 3). The sperm abnormalities were also significantly reduced in lipoic acid treated CBZ exposed rats as compared to CBZ exposed rats.

**Table 3**  
Effect of lipoic acid (LA) on selected sperm parameters and testicular steroidogenic machinery in carbimazole (CBZ)-exposed prenatates at their adulthood.

Parameter	Groups			
	Control <sup>s</sup>		CBZ-exposed*	
	Untreated	LA-treated	Untreated	LA-treated
Daily sperm count (millions/g testis)	13.56 <sup>a</sup> ± 3.14	12.47 <sup>a</sup> ± 1.65	8.95 <sup>b</sup> ± 0.94	11.76 <sup>c</sup> ± 1.22
<i>Sperm analysis (Epididymis)</i>				
Sperm count (millions/ml)	68.42 <sup>a</sup> ± 6.68	67.36 <sup>a</sup> ± 7.46	42.16 <sup>b</sup> ± 7.12	56.39 <sup>c</sup> ± 5.49
Sperm viability (%)	66.74 <sup>a</sup> ± 4.36	65.64 <sup>a</sup> ± 5.38	40.48 <sup>b</sup> ± 3.82	54.46 <sup>c</sup> ± 7.84
Sperm motility (%)	62.18 <sup>a</sup> ± 7.22	63.25 <sup>a</sup> ± 5.29	39.62 <sup>b</sup> ± 8.16	52.18 <sup>c</sup> ± 6.14
HOS Tail coiled sperm (%)	59.61 <sup>a</sup> ± 6.42	61.76 <sup>a</sup> ± 6.18	37.45 <sup>b</sup> ± 7.62	50.26 <sup>c</sup> ± 5.73
Abnormalities of sperm (%)	–	–	23.48 ± 6.38	10.76 ± 5.74
Pin shaped (%)	–	–	11	4
Rod shaped (%)	–	–	12	6
<i>Steroidogenic machinery (testis)</i>				
Total cholesterol (mg/g)	5.92 <sup>a</sup> ± 0.42	6.11 <sup>a</sup> ± 0.69	9.61 <sup>b</sup> ± 0.28	7.42 <sup>a</sup> ± 0.36
Testosterone (ng/ml)	6.47 <sup>a</sup> ± 0.21	7.12 <sup>a</sup> ± 0.36	3.27 <sup>b</sup> ± 0.33	4.81 <sup>c</sup> ± 0.18
3βHSD (nmol of NAD converted to NADH/mg protein/min)	16.28 <sup>a</sup> ± 3.92	15.76 <sup>a</sup> ± 2.69	8.69 <sup>b</sup> ± 2.36	12.46 <sup>c</sup> ± 1.86
17βHSD (nmol of NADPH converted to NADP/mg protein/min)	10.62 <sup>a</sup> ± 1.28	11.42 <sup>a</sup> ± 1.62	4.49 <sup>b</sup> ± 0.67	8.35 <sup>c</sup> ± 1.47
StAR mRNA (%) (n = 3) (Normalized against GAPDH band intensities)	100.66 <sup>a</sup> ± 1.15	101.33 <sup>a</sup> ± 0.57	84.33 <sup>b</sup> ± 2.08	92.33 <sup>c</sup> ± 1.527

Data are expressed as mean ± S.D. of 10 individuals.  
Comprise rats delivered to: <sup>s</sup>unexposed dams and \*dams exposed to CBZ during pregnancy.  
<sup>a-c</sup> Different superscripts in the same row indicate a significant difference (p < 0.05).

### 3.4. Testicular steroidogenic enzyme activity levels

Embryonic exposure to CBZ resulted in a significant increase in the testicular total cholesterol levels (62.33%) over controls (Table 3). A significant reduction in the activity levels of 3β- (46.62%) and 17β- (57.72%) HSDs were observed in the testis of CBZ exposed rats with respect to controls (Table 3). Further, gestational exposure to CBZ resulted in decreased expression levels of StAR mRNA in testis. On the other hand, substitution of lipoic acid to CBZ exposed rats resulted in the restoration of testicular steroidogenic parameters to control levels (Table 3).

### 3.5. Serum testosterone levels

Transplacental exposure to CBZ resulted in a 49.45% decrease in the serum testosterone levels as compared to controls (Table 3). On the other hand, serum testosterone levels were increased to control levels in CBZ exposed rats administered with lipoic acid. No significant changes in the serum testosterone levels were observed in rats of control group supplemented with or without lipoic acid.

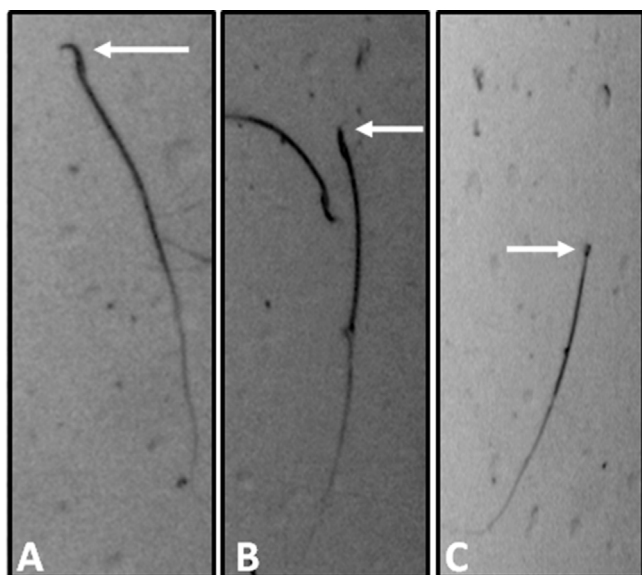


Fig. 2. Changes in sperm morphology of rats exposed to CBZ during the embryonic period Sperm head showing hook (A), rod shaped (B) and pin shaped (C).

### 3.6. Testis histological observations

The testis of control rats showed the normal architecture as evidenced by compactly organized seminiferous tubules with well developed germinal epithelium as basement membrane. The basal and adluminal compartments of seminiferous tubule showed successive stages of transformation of spermatogonia into spermatozoa (Fig. 3A). Transplacental exposure to CBZ resulted in marked degenerative changes of the testis as evidenced by loss of compactness of seminiferous tubules, fusion of tubules with ruptured epithelium, exfoliation of germ cells in the lumen of tubule and reduction of spermatogenic cells (Fig. 3B). On the other hand, a partial recovery of testicular architecture was observed in CBZ exposed rats substituted with lipoic acid (Fig. 3C). No marked differences were observed in the testicular architecture of control rats supplemented with lipoic acid as compared to controls (Fig. 3D).

### 3.7. Antioxidant enzymes, GSH and lipid peroxidation

The changes in selected enzymatic and non-enzymatic antioxidants and lipid peroxidation levels were shown in Table 4. Significant reduction in the activity levels of SOD, CAT, GPx and GR with a significant increase in the lipid peroxidation levels was observed in rats exposed to CBZ during the embryonic period as compared to controls. Testicular GSH content was significantly depleted in CBZ exposed rats as compared to controls. Administration of lipoic acid to CBZ-exposed rats resulted in the enhanced activity levels of SOD, CAT, GPx, GR and GSH content with a significant reduction in the lipid peroxidation levels in the testis as compared to CBZ-exposed rats. On the other hand, no significant changes were observed in the selected enzymatic and non-enzymatic antioxidants and lipid peroxidation levels in the testis of lipoic acid alone injected rats over the controls.

### 3.8. Molecular docking studies

Interestingly, the number of hydrogen bonds and the residues involved in the hydrogen bonding between the ligands (cholesterol, MMI and CBZ) and the modelled rat StAR protein were similar to that of selected ligands and the human StAR protein (Fig. 4). The magnitude of affinities between selected ligands and the StAR proteins of human and rat were as follows: CBZ > MMI > cholesterol. The details of DOPE scores along with the binding energies of selected ligands and the StAR proteins (human and rat) were given in the supplementary material.

## 4. Discussion

During the embryonic period, maternal thyroid hormones at least in part regulate the development of male reproductive tract and therefore, disturbances in these hormones may adversely affect the male reproductive health of the offspring's at their adulthood. The present study was designed to test this hypothesis. Our data revealed that the embryonic exposure to the antithyroid drug, CBZ adversely affects the male reproductive health in F1 rats at their adulthood as evidenced by the following: 1) reduction in the weights of testis and accessory sex organ indices, 2) deteriorated sperm quantity and quality, 3) diminished testicular steroidogenesis, 4) enhanced testicular oxidative stress, 5) disrupted testicular architecture and 6) reduced in vivo fertility ability.

The present work has shown that embryonic exposure to the

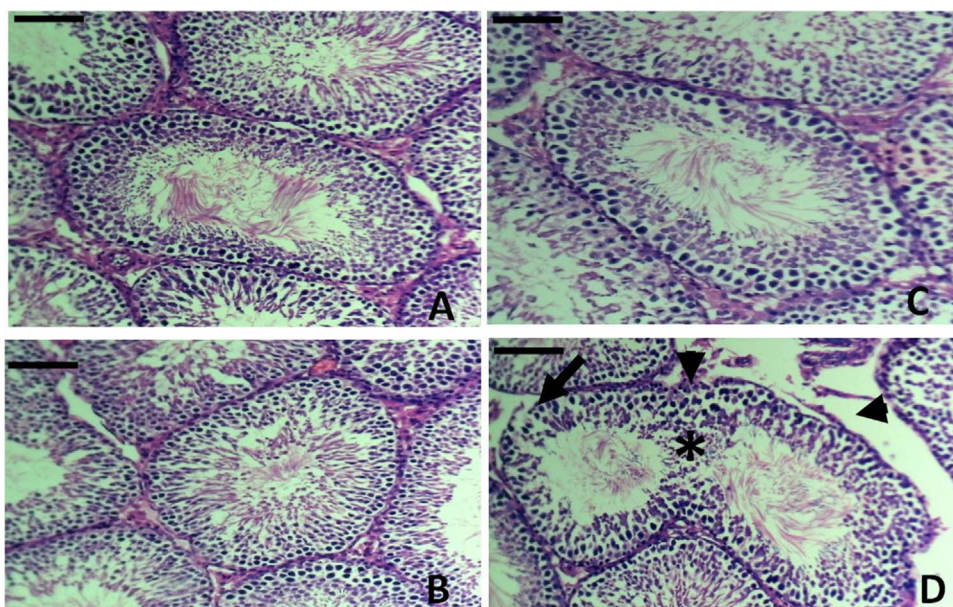


Fig. 3. Transverse sections of the testes in control and experimental rats (Scale bar 50 μm).

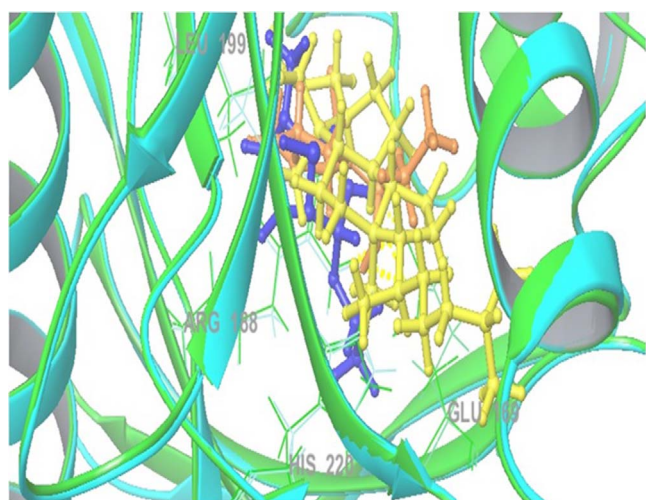
A: Photomicroscopic pictures of testis from control rat, shows compactly arranged seminiferous tubules with complete spermatogenesis.  
 B: Photomicroscopic pictures of testis from LA treated CBZ exposed rats, shows restoration of spermatogenesis with a decrease in exfoliation of germ cells in the lumen of seminiferous tubules;  
 C: Photomicroscopic pictures of testis from LA treated rats, shows normal structural organization of the testis;  
 D: Photomicroscopic pictures of testis from CBZ exposed rats, shows fusion of two seminiferous tubules with exfoliation of germ cells (asterisk) and ruptured epithelium (arrow).

**Table 4**

Effect of lipoic acid (LA) on selected testicular oxidative stress parameters in carbimazole (CBZ)-exposed prenatates at their adulthood.

Parameter	Groups			
	Control <sup>§</sup>		CBZ-exposed <sup>†</sup>	
	Untreated	LA-treated	Untreated	LA-treated
Lipid peroxidation (μmol of malondialdehyde formed/g tissue)	10.43 <sup>a</sup> ± 2.17	10.86 <sup>a</sup> ± 3.14	23.4 <sup>b</sup> ± 4.99	17.75 <sup>c</sup> ± 2.82
Superoxide dismutase (Units/mg protein)	0.66 <sup>a</sup> ± 0.074	0.68 <sup>a</sup> ± 0.052	0.22 <sup>b</sup> ± 0.071	0.53 <sup>c</sup> ± 0.043
Catalase (μmol of H <sub>2</sub> O <sub>2</sub> decomposed/mg protein/min)	0.51 <sup>a</sup> ± 0.018	0.49 <sup>a</sup> ± 0.021	0.21 <sup>b</sup> ± 0.017	0.43 <sup>c</sup> ± 0.024
Glutathione reductase (μmol of NADPH oxidized/mg protein/min)	3.87 <sup>a</sup> ± 0.087	3.89 <sup>a</sup> ± 0.12	1.75 <sup>b</sup> ± 0.092	3.52 <sup>c</sup> ± 0.083
Glutathione peroxidase (μmol of NADPH oxidized/mg protein/min)	2.81 <sup>a</sup> ± 0.125	2.92 <sup>a</sup> ± 0.103	0.92 <sup>b</sup> ± 0.151	1.74 <sup>c</sup> ± 0.135
Reduced glutathione (μmol of thiourea/mg protein/hr)	9.21 <sup>a</sup> ± 1.42	10.46 <sup>a</sup> ± 1.37	3.93 <sup>b</sup> ± 0.92	6.95 <sup>c</sup> ± 1.36

Data are expressed as mean ± S.D. of 10 individuals.

Comprise rats delivered to: <sup>§</sup>unexposed dams and <sup>†</sup>dams exposed to CBZ during pregnancy.<sup>a-c</sup> Different superscripts in the same row indicate a significant difference (p < 0.05).

**Fig. 4.** Molecular docking and interactions analysis of carbimazole (blue), methimazole (orange) and cholesterol (yellow) with residues Glu169, Arg188, Leu199, and His220 (A) Rat StAR (green) and human StAR (cyan) proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

carbimazole did not show any significant change in the body weight and relative weights of brain, liver, kidney, and spleen of rats indicating that the general metabolic condition of the animals was normal. It is well established that the structural and functional integrity of reproductive organs at least in part depends on the adequate supply of testosterone. In the present study, the relative weights of testes, and accessory sex organs (epididymis, seminal vesicles, vas deferens, prostate) was reduced in rats exposed to CBZ *in utero* [11,12]. This may reflect inadequate supply of androgens in CBZ exposed rats. Significant reduction in the testicular daily sperm count and the epididymal sperm parameters such as sperm count, sperm viability, sperm motility, and numbers of tail coiled sperms in CBZ exposed rats may suggest impairment of spermatogenesis and sperm maturation events. Our results are in agreement with previous reports who have demonstrated that the embryonic exposure to CBZ deteriorates sperm quality and suppressed spermatogenesis in rats at their adulthood [11,15]. The observed reduction in the weight of reproductive tissues and the reduced spermatogenesis could be due to deteriorated androgen biosynthesis and/or inadequate bioavailability of androgens in rats exposed to CBZ during the embryonic period. Consistently, we observed a significant reduction in the circulating levels of serum testosterone levels in CBZ exposed rats. It is well known that the biosynthesis of testosterone from the testis is comprised of several steps, including the channelling of cholesterol and the conversion of cholesterol to the testosterone. Steroidogenic acute regulatory protein (StAR) is a key protein involved in the transport of cholesterol from the outer to inner mitochondrial

membrane, while the conversion of cholesterol into the testosterone is catalyzed by 3β- and 17β HSDs [50,51]. The reduction in the activity levels of 3β- and 17β-HSDs and the expression of StAR mRNA levels in the testis of CBZ exposed rats may suggest improper channelling of cholesterol and reduced steroidogenesis. Accordingly, we recorded a significant increase in the total cholesterol levels in the testis of CBZ exposed rats. The results are in agreement with previous studies [6,9,52,53,11,12]. Surprisingly, *in silico* analysis revealed that the CBZ possess high affinity towards the human and the rat StAR proteins as compared to the cholesterol, an endogenous ligand of StAR and methimazole, the metabolite of CBZ [54]. Since, CBZ has the ability to cross the placental barrier; the direct inhibitory effect of CBZ on the testicular StAR proteins cannot be ruled out. However, from this study, it is not clear whether the carbimazole or its metabolite methimazole targets the testicular StAR proteins in the rats [54].

Our data also revealed that the fertility efficacy of CBZ exposed rats was reduced as compared to controls as evidenced by the absence of a copulatory plug from 4 out of 12 females cohabited with the CBZ-exposed rats. This may suggest compromised sperm fertility. The results are in agreement with previous studies [11]. Previously, it has been shown that the abnormal sperm fail to reach the site of fertilization and also to penetrate the zona pellucida of ova [55]. In this study, we found a significant increase in the sperm head abnormalities along with sperms with reduced motility in CBZ exposed rats over the controls. Embryonic exposure to the CBZ also resulted in the disruption of testicular architecture in the adult rats as evidenced by the ruptured epithelium, imperfectly organized interstitial connections, and most strikingly, the exfoliation of germ cells with lower sperm in the lumen of testis [11].

It is believed that testicular oxidant/antioxidant balance is considered important for male fertility and hence, testicular oxidative stress may adversely affect the male reproductive health [6,14,15,16]. Studies of Zamoner et al. [11] demonstrated that exposure of male rats to PTU during the perinatal period, adversely affects spermatogenesis, and testicular architecture accompanied by oxidative stress in the testis of rats. In general, the mammalian testes are equipped with the antioxidant enzymes such as SOD (EC: 1.15.1.1), CAT (EC:1.11.1.6), glutathione dependent enzymes (GPx: EC: 1.11.1.9 and GR: EC: 1.6.4.2) and non-enzymatic antioxidants including glutathione (GSH) [56,57]. SOD is involved in the dismutation of superoxides and CAT catalyzes the decomposition of peroxides. GPx utilizes GSH and negates the effects of peroxides in the testicular mitochondria, while GR is critical to sustain the ratio of reduced (GSH) to oxidized (GSSG) forms of glutathione in the tissues. GSH is a non-enzymatic antioxidant, which has the ability to quench oxygen-generated free radicals directly and also serves as the substrate for GPx activity [58]. The results achieved from this study, indicated a decline in the enzymatic (SOD, CAT, GPx and GR) and non-enzymatic (GSH) antioxidants with a significant increase in the lipid peroxidation levels in the testis of rats exposed to CBZ *in*

utero. This may suggest a failure of antioxidant defence system in the testis of CBZ exposed rats to counterattack the free radicals which in turn lead to the oxidative stress. Male gametes are very sensitive to oxidant/antioxidant changes and prolonged exposure of sperm cells to ROS may cause peroxidation of the cell membrane lipids and affects the sperm motility and viability. This, in turn, can affect the fertilizing potential of germ cells. The present results corroborate with the previous studies [6,8,14,15,16,51,59–61].

All the aforementioned reproductive ailments were restored in the lipoic acid plus CBZ exposed rats as evidenced by a significant increase in the weights of reproductive organs, improvement in the testicular steroidogenesis and spermatogenesis, reduction in the testicular damage and most notably, significant increase in the testicular activity levels of SOD, CAT, GPx and GR and GSH content. It is believed that the lipoic acid via its antioxidant property can scavenge several reactive oxygen species and also inhibits the generation of hydroxy radicals that attack sulphur-containing antioxidants [18]. Moreover, lipoic acid has ability to sustain the levels of protein thiols and modulate tissue endogenous antioxidants [18]. Based on these extraordinary properties, lipoic acid has been shown to be of interest as a therapeutic tool to protect the male reproductive health against a range of testicular toxicants in experimental animals, where oxidative stress is the part of underlying etiology [25,26,62,63]. Studies of the El-Maraghy and Nassar [64] reported that lipoic acid is as potent as the selenium in restoring the deteriorated testicular antioxidant status in rats exposed to the cadmium. Our results showed that, lipoic acid acts as a potent antioxidant capable of alleviating CBZ-induced testicular oxidative stress in adult rats. Further, it has been demonstrated that co-treatment of lipoic acid and TCDD to pregnant rats repairs fetal testicular steroidogenesis [20]. Therefore, the protective effects of the lipoic acid on male reproduction in CBZ exposed rats could be attributed to the antioxidant properties of lipoic acid or steroidogenic effects of lipoic acid and/or both.

Based on the findings, it can be concluded that the impairment of testicular spermatogenesis and steroidogenesis, and testicular oxidative stress may be responsible for the deteriorated male reproductive health in rats exposed to CBZ prenatally. Conversely, lipoic acid, which has emerged as a potential antioxidant and cell protecting agent negated the oxidative stress-mediated testicular toxicity and improved the fertility ability in CBZ exposed rats.

#### Conflict of interest

The authors declare that there is no conflict of interest regarding this manuscript.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxrep.2017.06.009>.

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