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Tartrazine, A Food Coloring Agent Exacerbate Streptozotocin-Induced Testicular and Epididymal Toxicity: A Study in Diabetic Rat Models

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KEYWORDS

Diabetes, tartrazine, oxidative stress, spermatogenesis, testosterone and rats

ABSTRACT:

The present study investigated the probable effect of a food coloring agent, tartrazine on male reproductive health in streptozotocin-induced diabetic rats. Healthy male rats were divided into three groups(n =8 per group) wherein rats in group I served as controls and rats in group II, and III were treated as diabetic rats which were injected with streptozotocin (STZ). In addition, rats in groups III wereorally administered with tartrazine (TTZ: 500 mg/Kg BW) over a period of 60 days. In diabetic rats, a significant reduction in the relative weights of reproductive organs, testicular daily sperm count, epididymal sperm count, sperm motility, sperm viability and sperm membrane integrity, circulatory levels of serum testosterone and the activity levels of testicular 3β- and 17β-hydroxysteroid dehydrogenases were observed over untreated rats. A significant increase in the circulatory levels of follicle stimulating hormone and luteinizing hormone in diabetic rats were observed over controls. Significant elevation in the lipid peroxidation levels and hydrogen peroxide content accompanied by a significant reduction in the activity levels of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in the testis of diabetic rats were observed over untreated rats. RT-qPCR analysis revealed that the expression levels of steroidogenic acute regulatory protein (StAR) mRNA was observed in the testis of diabetic rats. The testicular architecture was disorganized in STZ-induced diabetic rats. Interestingly, oral administration of TTZ further deteriorated the selected reproductive variables in STZ-induced diabetic rats as compared to STZ alone treated rats. Furthermore, the fertility efficacy of STZ plus TTZ administered rats was deteriorated as compared to STZ alone treated rats. In silico analysis also indicated that TTZ can able to occupy the same ligand binding pocket and compete with cholesterol, an endogenous ligand for StAR protein thereby interferes with testosterone biosynthesis. From the results, it can be concluded that the administration of TTZ accelerate the adverse on testicular functions in STZ-induced diabetic rat models.

1. Introduction

Diabetes mellitus (DM) is one of the most common and prevalent chronic metabolic disorder which is identified as a biggest epidemic disease in 21st century [1] Many studies have shown that several factors such as heredity aspects, environmental factors and modern lifestyle habits including consumption of alcohol, and food habits including fatty foods, junk foods and sugar sweetened drinks could be plausible reasons for the DM [2-4]. Almost all the vital organs including male reproductive tract are vulnerable targets of both type 1 and 2 DM [5, 6]. With regards to the male reproduction, DM negatively affects sperm number, sperm motility, testosterone levelsand also testicular oxidative stress [7-10] in experimental models, suggesting inhibition of testosterone and triggered testicular and epididymal oxidative stress could be one of the plausible reasons for the deterioration of male reproductive health [11].

Azo dyes are widely used in food products to obtain flavour and colour thereby to attract and enhance human

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consumption. However, consumption of food containing azo dyes may be associated with several complications including hepatic cancer, and nephritic failure [12] Tartrazine [TTZ: E 102, FD, and C Yellow)] is one of the widely used synthetic azo dyes in the food industries related to food preparations like juices, sauces, ice-creams is well known and also in the preparations of cosmetics, shampoos and medications. Published studies have shown that the usage of TTZ may cause several adverse effects including angioedema, asthma, and urticaria, genotoxic and mutagenic, immunotoxic effects and disorders of nervous system [13-17]. Human studies also indicated that the consumption of TTZ may be harmful as it causes thyroid cancer, genotoxicity, asthma, liver and kidney abnormalities [18,19]. Previously, our findings and others have shown that the administration of TTZ deteriorates male reproductive tract functions via suppression of testosterone synthesis associated withtesticular oxidative damage in rats as animal models [20-22]. The findings of these studies question the usage of TTZ in food products. Moreover, in developing countries like India, the usage of TTZ in food products exceeds acceptable daily intake [23]. Interestingly, children and the population who are at reproductive age seems to be vulnerable targets to the TTZ, as these age groups of the population are attracted to the colored food products and thus, are the major consumers. Therefore, the health risk assessment studies especially, male reproduction are immediately required to analyze the safety of TTZ. Further, as DM negatively affect male fertility efficacy and TTZ can able to trigger DM and suppress male reproductive health, it is conceivable that both DM and TTZ may be considered a double jeopardy with regard to male reproductive health. Thus, assessment of DM and TTZ on male reproduction enable plausible
mechanisms which at a later stage might be helpful to develop therapeutic strategies. Therefore, the central objective of present study was aimed to investigate the probable effect of TTZ on male reproductive health in STZ-induced diabetes in rats as experimental models.

2. Materials and Methods

Chemicals

Streptozotocin (STZ) (Figure 1A), NAD, NADPH, INT, glutathione reductase, reduced glutathione and oxidized glutathione were purchased from Sigma Chemicals Pvt. limited, Missouri, USA. Tartrazine (TTZ) (Figure 1B) and other chemicals used for the work were purchased from Himedia Chemicals Pvt. limited, Bangalore, India.

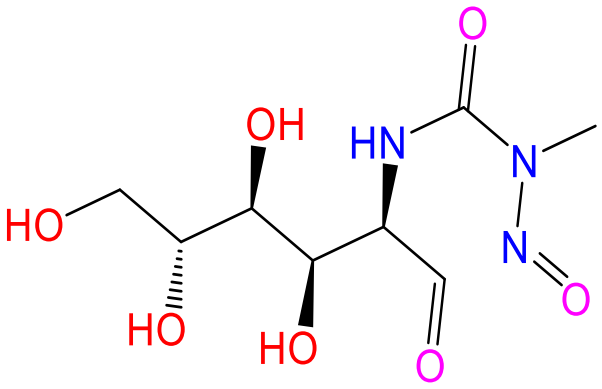

Figure 1A. Structure of Streptozotocin

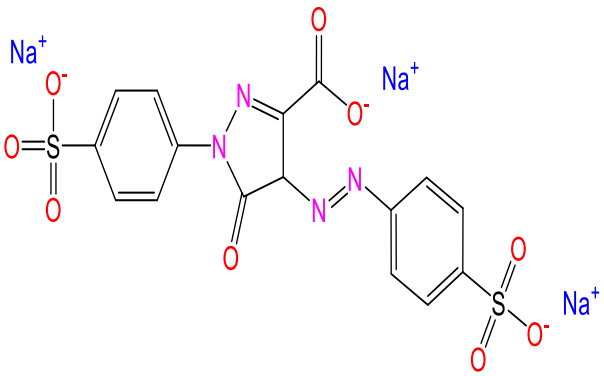

Figure 1B.Structure of Tartrazine

Maintenance of animals

Healthy male adult Wistar albino rats (200 ± 10 gm) were purchased from Sri Venkateswara enterprises, Bangalore. The rats were placed in polypropylene cages lined with sterile paddy husk, maintained under controlled conditions (Temp: 23±2ºC; Humidity: 50±5%; 12 h light: 12 h dark) in the animal house at Sri Padmavati Mahila Visvavidyalayam, Tirupati. Rats were fed on pellet feed and water ad libitum. All experiments were carried out in accordance with the guidelines of the CPCSEA [24] and the procedures approved by the Institutional Animal Ethical Committee, Sri Padmavati Mahila Visvavidyalayam (Women's University No.

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1677/PO/Re/S/2012/CPCSEA/IAEC-05; Dt/23/12/2014).

Experimental Design

Rats were randomly divided into three groups of eight animals each. Rats in first group (n=8) served as controls and animals in group II and III (n=16) were treated as experimental groups. Rats in group II were injected with streptozotocin (STZ) at a dose of (50mg/Kg BW) to induce diabetes [25], while rats in group III received both STZ (50 mg/Kg BW) and tartrazine (500 mg/Kg BW via gavage) (TTZ) over a period of 60 days. The time interval 60 days was selected to elucidate the effect of test chemicals on one complete spermatogenic cycle in rats [26].

Fertility studies

After completion of treatment, control group animals and experimental group animals were allowed to mate with normal female rats bears a normal cycle on 1: 1 ratio to assess fertility capacity. Everyday female rats were checked for the appearance of sperms or vaginal plugs. Female rats having the vaginal plugs or sperms in the vaginal fluid was said to be pregnant and separated and the day was referred as 0 gestation day. To determine the corpora lutea of each rat, pre implantation loss of in rats the six animals from the control group and experimental group were sacrificed on 6^{th} day of gestation period. On the 18^{th} day of the gestation period of the pregnant animals, they were laparotomized to record the post implantation loss and live foetuses.

Collection of Blood

Following the fertility analysis, both control and experimental rats were fasted overnight, and the blood was collected through cardiac puncture from the rats. The blood was separated into two parts from each animal wherein one part was used for the analysis of glucose levels in both control and experimental groups, and the other part was used to separate the serum from blood and preserved at -20˚ C for hormonal analysis.

Body and reproductive organ weights

Before sacrifice, the body weights of rats were recorded and were sacrificed humanely by cervical dislocation. Immediately the animals were dissected, reproductive organs were isolated extra tissue around the organs was chopped off, cleared the blood adhered to the tissues and weighed to their nearest milligram using an electronic weighing balance (Shimadzu- BL -220 H, Japan). Tissue somatic indices were calculated using the standard formula:

Tissue somatic index = Weight of the tissue in grams/ Rat weight in grams×100

Sperm Parameters

Daily sperm production was determined using the method described by Blazak et al. [27]. The testis was homogenized in 50 mL of normal saline containing 0.01% Triton X- 100 and allowed for 60 sec. The total number of sperm heads was enumerated using a haemocytometer after proper mixing of each sample. The total number of sperms produced for each gram of testis per day was evaluated and expressed in millions/gram tissue [28]. The epididymis was minced into pieces in the saline at 37º C to obtain the seminal fluid and used to analyze the sperm count, motility prescribed by Belsey et al. [29]. The total number of sperms and motile sperms was counted using the haemocytometer and were expressed in millions/mL. The viable sperms ratio to the dead sperms was calculated using trypan blue (1%) solution [30] and sperm viability was expressed as percentage of unstained sperm of the total sperm counted. The integrity of the sperm membrane was determined by the method Jayendran et al. [31]. The sperms were subjected to hypo-osmotic swelling (HOS) solution and observed for the coiled sperms under phase contrast microscope (Olympus, Olympus Optical Co.Ltd. and the percentage of coiled sperms was calculated.

Oxidative stress parameters

A10% w/v testis homogenate was prepared with sodium phosphate buffer (10mM) using the homogenizer. Using the standard protocol the sub cellular fractions were separated under the cold temperature. The levels of lipid peroxidation were determined by the method of Ohkawa et al. [32] and data resulted was expressed as µmol of MDA/gm tissue. The levels of hydrogen peroxide were estimated according to the method described by Pick and Keisari [33], and the quantity of hydrogen peroxide formed was expressed as nmol hydrogen peroxide produced/mg protein/min. The activity levels of superoxide dismutase were determined by Marklund and Marklund [34]. The activity levels of

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superoxide dismutase were expressed in nmol of pyrogallol oxidized/mg protein/min. The activity of catalase was estimated according to the method of Claiborne [35]. The units of the catalase activity levels were expressed as nmol of hydrogen peroxide metabolized/mg protein/min. The activity levels of glutathione peroxidase were determined by the method Paglia and Valentine [36]. The units of the glutathione peroxide activity levels were expressed as nmol of NADPH oxidized/mg protein/min. The activity of glutathione reductase was described by Carlberg and Mannervik [37] and units of the activity levels of glutathione reductase were expressed as nmol of NADPH oxidized/mg protein/min.

Assay of testicular steroidogenic enzymes

Decapsulated testes were homogenized (10%W/V) in Tris-Hcl (20mM) buffer (pH 6.8) then separated the microsomal fraction using centrifugation and used as a source for enzyme. The activity levels of 3 β- hydroxy steroid dehydrogenase (3β-HSD) and 17β-hydroxy steroid dehydrogenase (17β-HSD) were determined by the method of Bergmeyer [38]. Briefly, 2mL reaction mixture prepared with 0.5µmol NAD cofactor for 3β- HSD and NADPH cofactor for 17β-HSD, 100µmol of sodium phosphate buffer (pH 9.0) and 0.8µmol of dehydroepiandrosterone substrate for 3β-HSD and androstenedione substrate for 17β-HSD with 100µl an enzyme source separately for both. The absorbance of the samples was measured at 340nm at 20 sec time intervals for 180 sec using UV-Visible spectrophotometer. The units were expressed as nmol of NAD converted to NADH/mg/protein/min for 3β-HSD and nmol of NADPH converted to NADP/mg/protein/min for 17β- HSD.

Estimation of serum hormones concentration

The activity levels of testosterone, FSH and LH 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 hormonal assays in this method is about100 pg/ml.

StAR protein expression using RT-PCR

RNA was isolated from the rat testis using the Gene JET RNA isolation kit (Thermo Scientifics) according to the manufacture protocol. Thirty milligrams of testis was taken and homogenized using motor and pestle by adding the liquid nitrogen and immediately shifted to a 1.5 mL microcentrifuge which contained 300µl of a lysis buffer and β-mercaptoethanol mix the contents, 600 µl of proteinase K was added, vortex the contents, and incubated for 10 min at 15-25º C. After the incubation the mixture was centrifuged for 10 min at 12000 rpm and the supernatant was separated into a new sterile RNase-free microcentrifuge. To the supernatant, 450µl of 96-100% ethanol was added and properly mixed and extracted the RNA by following protocol in the kit and used for the cDNA synthesis. By using the cDNA synthesis kit (Thermoscientific, K1621) protocol the reaction mixture was made and mixed thoroughly and kept for incubation at 42º C for one hour. The termination of reaction mixture was done by heating the reaction mixture for 5 min at 70º C. The product of the reverse transcription was used for PCR. PCR reactions having a primary denaturation of 94˚C for 4 minutes, followed by the 35 enlargement cycles for denaturation at 94˚C for 30 seconds, annealing at 53.3˚C to StAR and 54.4˚C to GAPDH for 45 seconds and with extension at 72˚C for 120 seconds. The last extension step set at 72˚C for six minutes and succeeded by the cooling at 4˚C. Subsequently after PCR amplification, a series of reactions was transferred on to the gel (1.8%) made of agarose having ethidium bromide in order to observe the gel under the UV Gel Doc. The levels of StAR mRNA expression were normalized versus GAPDH.

Molecular Docking Analysis

In the present study, the rat StAR protein was modeled by selecting human StAR protein template available in the (Protein Data Bank [PDB] ID: 3P0L). The modeling, validation and ligand pocket analysis of rat StAR protein was previously described Vasudha et al. [39]. The ligands used in this study were cholesterol (endogenous ligand) and the test compounds STZ and TTZ. The structures of cholesterol (CID: 5997), STZ (CID: 29327) TTZ (CID: 164825) were downloaded from PubChem database in SDF format. The chemicals in SDF format were converted into PDB format using

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Chimera software and docked against the modeled rat StAR protein and human StAR protein AutoDock4 with following parameters: Lamarckian genetic algorithm (numbers of GA Runs, 10; population size, 150; maximum number of energy evaluations, 2500000; number of generations, 27000; rate of gene mutation, 0.02; rate of cross over, 0.8) was used as a genetic algorithm parameters. The docked protein-ligand structures were subjected to Ligplot analysis to know the interactions between the StAR protein and selected ligands.

Histological study of testis tissue

Immediately after isolation, testes from control and experimental group rats were shifted to a Petri dish and washed with normal saline. Later each testis was fixed in the Bouin's fixative for 24h, dehydrated in ascending series with alcohol, cleared in xylol, and were embedded in the paraffin wax. Thin sections of thickness with 6µm were made, stained using hematoxylin subsequently with eosin stain [40] and histopathological changes were observed under the Olympus microscope.

Estimation of Protein

The estimation of the protein content in the enzyme source was determined by using Lowry et al. [41] method with bovine serum albumin as standard.

Results

The control, STZ and STZ plus TTZ rats were monitored carefully throughout the experimental time period for any signs of clinical symptoms. In this study no mortality and no significant changes in the general appearance, activity and body positions were observed in any of the experiment and control groups. Further, none of rats was excluded from experiments.

In general, all the rats from control and experimental groups exhibited signs of sexual motivation including licking the female genetalia, chasing the females, and attempted mounting trials numerous times as soon as the female was introduced. Mating index and fertility index was evaluated to determine the ability of male rats to impregnate the female rats in a given time interval. While no significant differences in the mating index was observed in any of the control and experimental rats, the fertility index in STZ treated (68%), and STZ + 500 mg TTZ (59%) was reduced as compared to controls (100%). A significant increase in the mean pre-implantation loss in females cohabited with STZ plus 500 mg TTZ (46%) as compared to STZ alone treated (13%) and control (6%) rats (Figure 2A). Similarly, a significant increase in the mean post-implantation loss in females cohabited with STZ plus 500 mg TTZ (34%) as compared to STZ alone treated (12%) and control (6.17%) rats (Figure 2B). The body weights of fetuses were not significantly different in experimental groups over controls. Furthermore, no significant external (polydactyly, syndactyly, drooping wrist and anogenital distance) and teratogenic (fusion of ribs, sternebrae, frontal, parietal, intraparietal, tarsal and claws) abnormalities were observed in fetuses of rats cohabited with STZ, STZ plus TTZ male rats (Table 1).The mean value of the blood glucose levels in untreated rats was 99.4±12.32 mg dL-1. Whereas, a significant ($p< 0.05$) increase in the blood glucose levels was observed in STZ treated animals (228.67%) as compared to control animals.

Table1: Effect of tartrazine (TTZ) on fertility studies in diabetes induced (STZ) **male rats**

Parameter	Control	STZ	STZ+TTZ 500mg
Conception time (Days) #	1.60[a]±0.42	3.10 [b]±0.62 (93.75)	5.24 [d]±0.82 (69.03)
Mating index (%)	100	100	100
Fertility index (%)	100	68.74	56.81
No. of corpora lutea/ rat*	14.43[a]±0.39	13.39 [b]±0.36 (-7.20)	12.56 [d]±0.29 (-6.19)

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No. of Implantations/rat*	13.23 [a] ±0.67	6.24 [b] ±0.49 (-52.83)	5.32 [d]±0.41 (-14.74)
Pre- implantation loss (%)	5.84	13.07	46.23
Post- implantation loss (%)	6.17	12.42	34.28
Live fetuses/rat*	12.82[a]±1.31	9.51[b]±1.24 (-25.81)	4.96 [d]±1.38 (-61.31)
Weight of the fetuses(g)	5.82 [a]± 0.29	3.91 [b]±0.31 (-32.81)	2.18 [d]±0.32 (-62.54)

Values are mean ± S.D. #n=16; n=8; 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 < 0.05

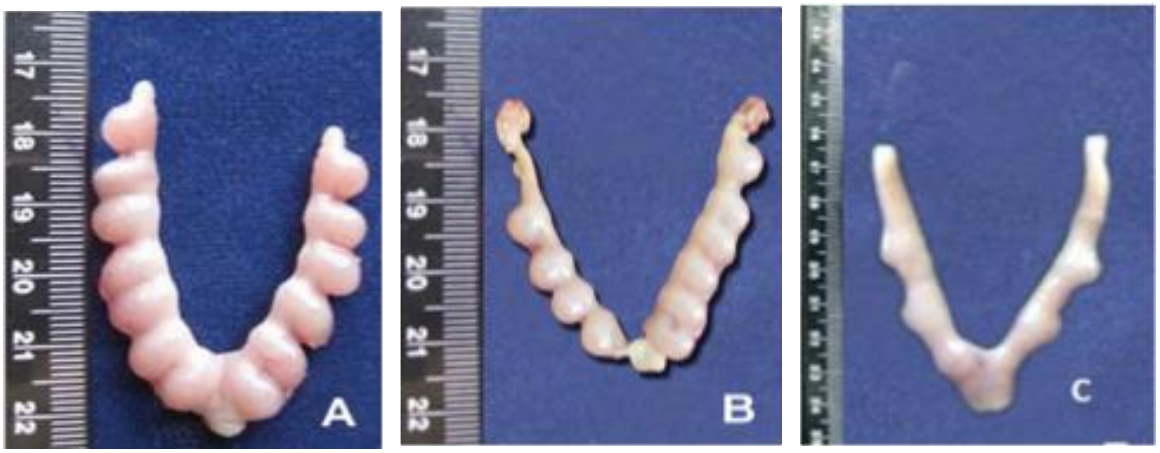

Figure 2A A: Uterus of pregnant rat (normal female mated with control I male rat) showing implantations

(13.23±0.67) on 6th day of pregnancy.

B:Uterus of pregnant rat (normal female mated with male rat treated with STZ) showing implantations

(9.51±1.24) on 6th day of pregnancy.

C:Uterus of pregnant rat (normal female mated with male diabetes-induced rat treated with 500mg TTZ)

showing implantations (5.28±0.38) on 6th day of pregnancy.

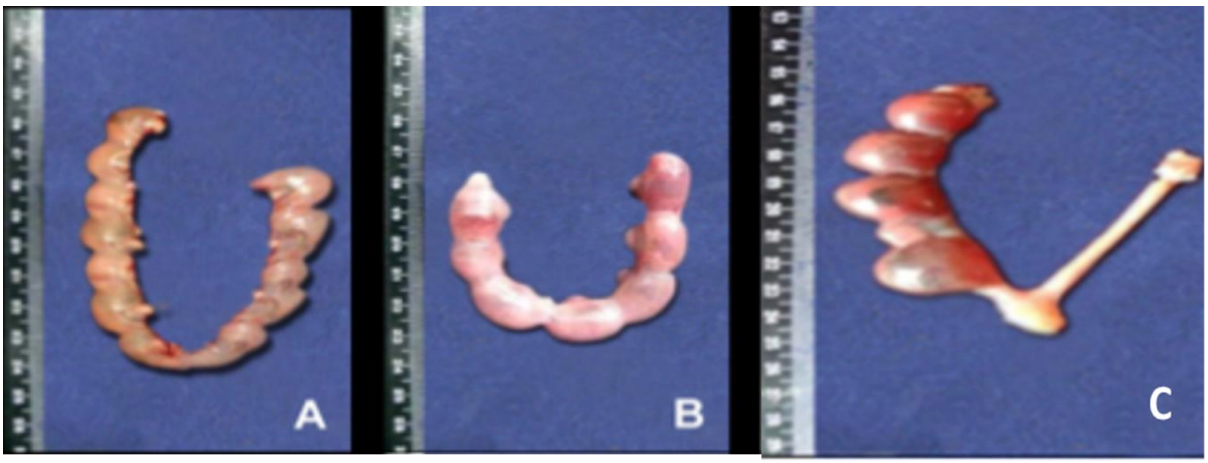

Figure 2B: A:Uterus of pregnant rat (normal female mated with control I male rat) showing implantations

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(12.82±1.31) on 18th day of pregnancy.

B: Uterus of pregnant rat (normal female mated with male rat treated with STZ) showing implantations

(6.24±0.49) on 18th day of pregnancy.

A further increase in the blood glucose levels was observed in STZ rats treated with 500 mg/Kg BW TTZ (297.02%) (Figure 3).The body weights and weights of

C:Uterus of pregnant rat (normal female mated with male diabetes-induced rat treated with 500mg TTZ)

showing implantations (4.96±1.38) on 18th day of pregnancy.

the reproductive organs of the control and experimental animals are presented in Table 2.

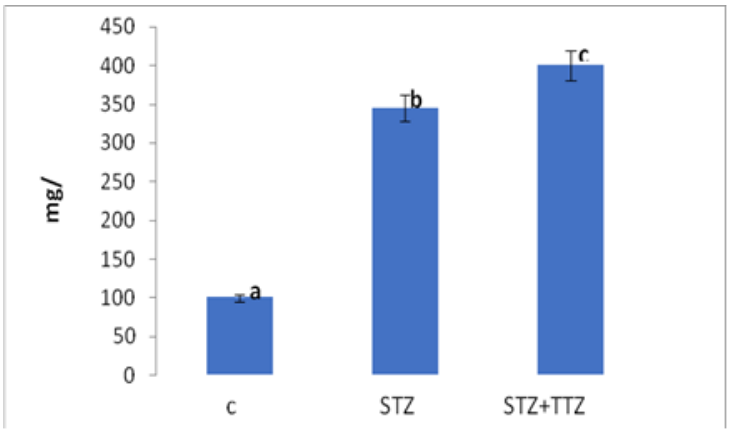

Figure 3:Effect of tartrazine (TTZ) on the blood glucose levels in diabetes induced (STZ) **rats**

Values are mean ± S.D. of 8 individuals for each treatment group Bars with different superscripts are significantly differ from each other at $p<0.05$

Table 2: Effect of tartrazine (TTZ) on body weights and reproductive organ weights in diabetes induced (STZ) **male rats**

Parameter	Control	STZ	STZ+TTZ 500mg
Body weights	310.5 [a]±10.12	265.2 [b]±11.02	271.8 [d] ±11.65
		(-14.58)	(-12.46)
Testis	1.10 [a] ±0.05	0.84 [b] ±0.02	0.69[d] ±0.01
		(-23.63)	(-37.27)
Epididymis	1.19 [a] ±0.10	0.94. [b]±0.08	0.68 [d]±0.03
		(-21.00)	(-42.85)
Seminal vesicle	0.51 [a] ±0.06	0.37 [b] ±0.03	0.23 [d]±0.01
		(-27.45)	(-54.90)
Prostate gland	0.19 [a] ±0.02	0.11 [b] ±0.01	0.07 [f]±0.01
		(-42.10)	(-63.15)

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

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

Values with different superscripts in a row differ significantly from each other at $p< 0.05$

The mean body weight of animals in control group was 320.26±12.24. Significant ($p<0.05$) decrease in the mean body weights was observed in STZ treated animals (-17.19%) when compared to the controls. Further significant ($p<0.05$) decrease in the body weights of the 500mg/Kg BW TTZ (-17.08%) over STZ treated animals. The reproductive organ indices of rats from control and experimental groups indicated that the relative weights of testes, epididymis, seminal vesicles, vas deference and prostate gland were significantly ($p<0.05$) decreased in STZ treated animals as compared to controls. The decrease in the relative weights of reproductive organs was further noticed in rats subjected to both STZ and TTZ as compared to STZ alone treated rats. The total number of sperms (-25.33%), viable sperms (-22.22%), motile sperms (-17.89%) and HOS tail coiled sperms (-18.81%) were significantly ($p<0.05$) decreased in STZ treated animals over controls. An additional decline in the sperm parameters was observed in the STZ rats treated with 500mg/Kg BW TTZ (-43.65%, -48.37%, -43.65% and -40.86%) (Table 3). The mean circulatory level of testosterone in the rats of control group was 2.92±0.26 pg/ml. The average serum testosterone level was significantly ($p<0.05$) decreased in STZ treated rats (-34.24%) as compared to controls. The circulatory levels of testosterone were further reduced in the STZ rats treated with 500mg/Kg BW TTZ (-24.47 %) when compared with STZ induced rats. In contrast, significant ($p<0.05$) increase in the levels of FSH (75.92%) and LH (160.32%) was observed in STZ treated animals. Surprisingly, TTZ treatment at selected doses resulted in further decrease in the levels of testosterone associated with an increase in serum gonadotropins FSH (47.04%) and LH (25.46%) (Figure 4).

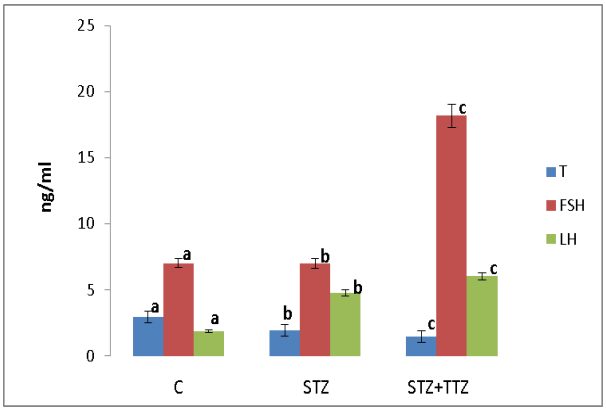

Figure 4: Effect of tartrazine (TTZ) on the testosterone, FSH and LH in diabetes induced (STZ) rats.

The activity levels of 3-β HSD (Figure 5), 17- β HSD (Figure 5) and StAR mRNA expression levels (Figure 6) were significantly ($p<0.05$) decreased in the testes of the STZ treated rats over controls. Further significant ($p<0.05$) decline was observed in the activities of steriodogenic enzymes and the expression levels of StAR mRNA in testes of STZ rats treated with TTZ as compared to controls (Figures 5 and 6).

The testicular architecture in STZ and STZ plus TTZ treated rats were deteriorated as compared to control rat testis. A compact arrangement of the seminiferous tubules with intact epithelium as basement membrane was clearly observed in untreated rats, suggesting intact testicular architecture.Moreover, the lumen was completely occupied by spermatozoa in the testis of control rats (Figure 7A). The lack of compactness of seminiferous epithelium and disrupted epithelial membrane were conspicuous in the testis of experimental rats.

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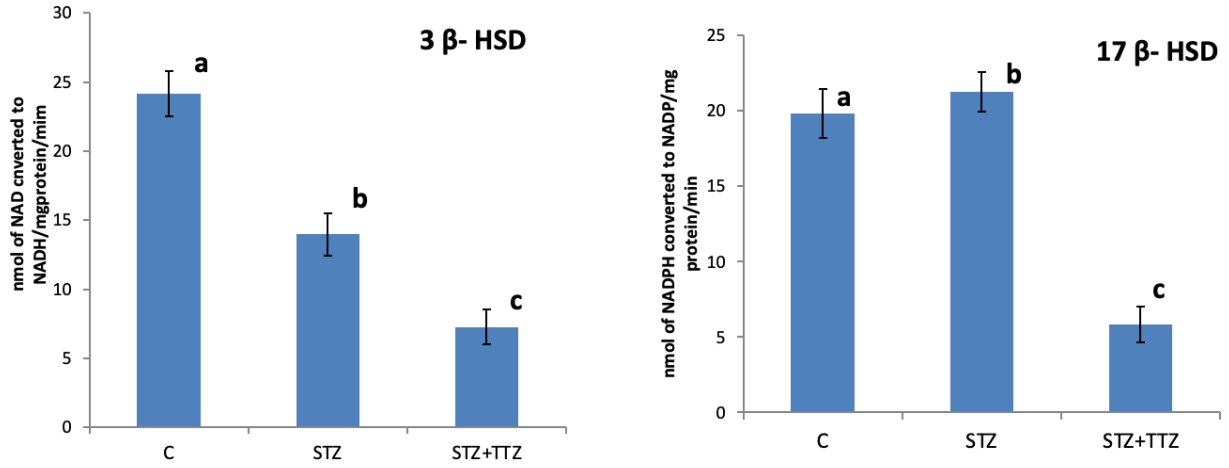

Figure5: Effect of tartrazine (TTZ) on activity levels of steroidogenic marker enzymes in testis of iabetes induced (STZ) male rats.

Moreover, the lumen of the seminiferous epithelium showed reduced spermatozoa in STZ, (Figure 7B) and STZ plus TTZ (Figure 7C) rats, suggesting reduced production of sperm.

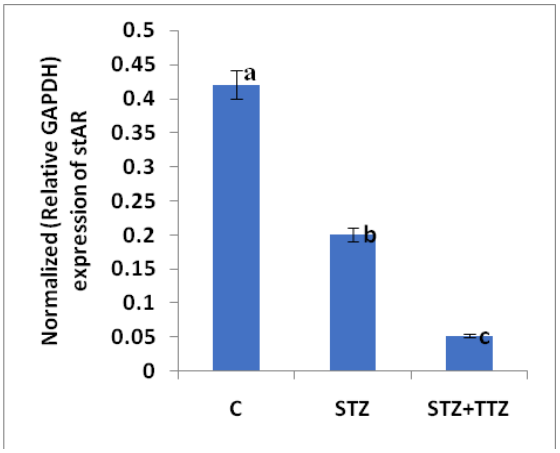

Figure 6: Effect of tartrazine on the mRNA levels of StAR gene expression in testes of diabetes induced (STZ) male rats.

The levels of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were significantly ($p<0.05$) decreased in STZ treated animals when compared to controls. Conversely elevated levels of lipid peroxidation and hydrogen peroxide content were observed in the testis of STZ rats over control rats. The levels of antioxidant enzymes were further reduced with increased levels of lipid peroxidation and in the STZ rats treated with TTZ at selected concentrations (Table 4) as compared to STZ treated rats.

Molecular docking studies were performed to know the interactions between the STZ and StAR protein. The binding energies and the amino acids that were involved in the interactions between the StAR and cholesterol and StAR and STZ and StAR and TTZ were shown in Table 5 and Figure 8. The binding energy between the modelled rat StAR protein and cholesterol and human StAR protein and cholesterol was -8.23 kcal/ mol and -8.17 kcal/mol respectively. Interestingly among the experimental chemicals, the binding affinity of TTZ was greater than the STZ towards the modelled rat and human StAR proteins. The binding energy between the modelled rat StAR protein and TTZ and human StAR protein and

cholesterol was – 7.21 kcal/ mol and -7.38 kcal/mol, respectively as compared to the binding energy between the modelled rat StAR protein and STZ (-6.08 kcal/ mol) and human StAR protein and STZ (-6.72 kcal/mol). Interestingly, most of the amino acids involved in interactions between the ligands (cholesterol, STZ, and TTZ) – StAR proteins (modelled rat StAR protein and human StAR protein) were similar (Table 4).

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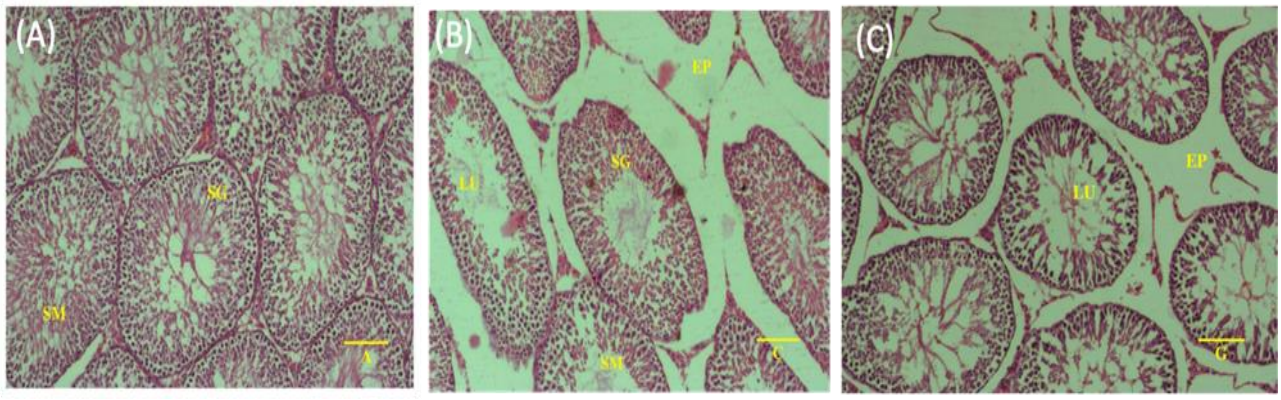

Figure 7: Photomicrographs of testicular architecture of control rats (A) and diabetic rats (STZ) (B) and diabetic rats (STZ) treated with 500mg/Kg BW Tartrazine (C). Sperm Mass (SM), Interstitial spaces (IS), Seminiferous tubules (ST), Lumen (LU), Spermatogonia (SG), Epithelium (EP) Scalebar=20µm.

Table 3: Effect of tartrazine (TTZ) on sperm parameters in diabetes induced (STZ) male rats.

Parameter	Control	STZ	STZ+TTZ 500mg
Daily sperm production (millions/g testes)	22.5 [a] ± 1.7	16.10[b] ± 1.21 (-28.44)	10.21 [d]±1.13 (-54.62)
Sperm count (millions/mL)	75.16[a] ± 7.02	56.12[b] ± 6.12 (-25.33)	42.35[d]±3.26 (-43.65)
Motile sperm (%)	77.36[a] ± 4.61	63.52[b] ± 4.68 (-17.89)	48.32 [d]±3.12 (-37.53)
Viable sperm (%)	76.54[a] ± 5.68	59.53[b] ± 4.12 (-22.22)	39.51 [d] ± 2.65 (-48.37)
HOS- tail coiled sperm (%)	65.21[a] ± 3.95	52.94[b] ± 2.65 (-18.81)	38.56 d ±2.13 (-40.86)

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

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

Values with different superscripts in a row differ significantly from each other at $p < 0.05$

Table 4: Effect of tartrazine (TTZ) on pro and anti-oxidant enzyme levels in diabetes induced (STZ) male rats

Parameter	Control	STZ	STZ+TTZ500mg
Hydrogen peroxide (nmol per mg protein per min)	10.95 [a] ±1.81	17.52 [b] ±1.38 (62.03)	27.36 [d] ± 1.95 (149.86)
Lipid peroxidation(µ molmalondialdehyde per g	11.68 [a] ±1.09	18.96 [b] ±1.12 (62.32)	25.56[d] ± 1.54 (118.83)

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tissue)			
Super oxide dismutase(nmolpyrogallol oxidized per mg protein per min)	**7.15 [a] ± 0.62**	**3.10 [b] ± 0.05 (-56.64)**	**2.05 [d] ± 0.01 (-71.32)**
Catalase(nmol H_2O_2 metabolized per mg protein per min)	**16.52 [a] ±1.82**	**11.83[b] ± 1.16 (-28.38)**	**9.36 [d] ± 1.09 (-43.34)**
Glutathione peroxidase (nmol NADPH oxidized per mg protein per min)	**42.73 [a] ±1.97**	**25.16 [b] ± 1.63 (-41.11)**	**17.27 [d] ± 1.29 (-59.58)**
Glutathione reductase (nmol NADPH oxidized per mg protein per min)	**47.26 [a] ±4.32**	**28.92 [b] ± 3.08 (-38.80)**	**18.92 [d] ± 2.19 (-59.96)**

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

Values with different superscripts in a row differ significantly from each other at $p < 0.05$

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

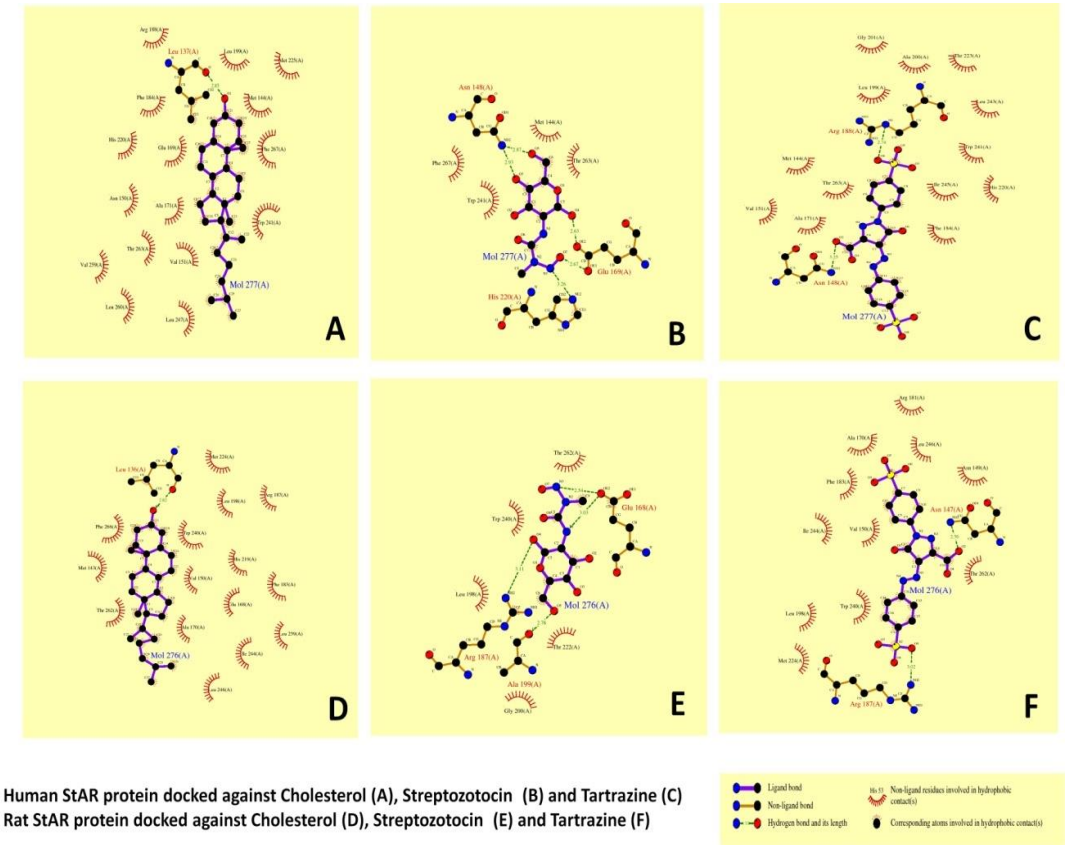

Figure 8: Molecular docking interaction of StAR protein with Strptozotocin, tartrazine and Cholesterol

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Table 5: Binding affinities of StAR protein amino acids interaction withcholesterol, streptozotocin and tartrazine

Ligand	Binding affinity
Cholesterol	-8.23
Streptozotocin	-7.21
Tartrazine	-6.08

Discussion

Rats subjected to STZ exhibited signs of reproductive toxicity as indicated by reduced reproductive organ weights, poor sperm quality and quantity, reduced testicular steroidogenesis, and testicular oxidative stress. In the current study, spermatotoxic effects caused by STZ could be ascribed to impaired testicular spermatogenesis and post-testicular events. Earlier studies observed that STZ administration caused reduced levels of daily sperm production, epididymal sperm count, motile, viable and HOS-tail coiled sperms in rat models [8, 42] . Reports of Lei et al. [43] indicated that STZ administration led to improper spermatogenesis and epididymal sperm maturation events. It is well accepted that the structural and functional integrity of reproductive organs at least in part depends on the adequate supply of androgens [44] Testosterone biosynthesis comprises of several steps and the key steps include channelizing cholesterol from the outer to inner mitochondrial membrane through steroidogenic acute regulatory protein and its metabolism via the activities of 3βHSD, and 17βHSD. In rats, subjected to both TTZ and STZ a significant reduction in the expression levels of StAR mRNA associated with a significant reduction in the activity levels of steroidogenic marker enzymes, 3 β-HSD and 17β-HSD was observed in the testis as compared to STZ alone treated rats, suggesting improper channelling of cholesterol across the mitochondrial membrane of testis in experimental rats and the reduced activity levels of 3 β-HSD and 17β-HSD might suggest improper metabolism of cholesterol. These findings might augment the adverse effects of TTZ in the modulation of Leydig cell steroidogenesis in STZ treated rats. FSH and LH are the two important pituitary gonadotropins that control and coordinate spermatogenesis and steroidogenesis respectively [42]. A significant elevation in the circulatory levels of serum gonadotropins FSH and LH in rats subjected to both TTZ and STZ as compared to STZ alone treated rats might reflect enhanced lack of responsiveness of testis in rats subjected to both STZ and TTZ as compared to their respective controls. Based on the health risks associated with the STZ and TTZ in humans and also based on the inhibition of testosterone biosynthesis in rats subjected to both STZ and TTZ, an attempt has been made to know whether STZ and TTZ could interfere with StAR protein using molecular docking approach. The results indicated that the amino acids involved in the interactions between the STZ and TTZ against human StAR and modelled rat StAR protein was similar. The results obtained from this study at least in part demonstrate that the STZ and TTZ could interfere with the transcription (PCR studies) and translation of StAR (*In silico* analysis) via disruption of cholesterol channelling across testicular membranes thereby reduced testosterone production in rats. Whether similar interactions between the amino acids of human StAR and modelled rat StAR proteins and the ligands, STZ and TTZ might cautions the usage of STZ and TTZ in humans.

It is believed that oxidative stress is one of the major culprits for the deterioration of male reproductive health. In general, testis is equipped with endogenous enzymatic and non-enzymatic antioxidants to control excessive generation of free radicals [45]. However, testicular oxidative stress occurs due to a failure to counterattack the overwhelming free radicals by antioxidants. Among the antioxidant enzymes, SOD, CAT, GPx and GR play an important role in the protection of testis against free radical induced oxidative toxicity. SOD and CAT are mainly involved in the dismutation of superoxide and removal of hydrogen peroxide, respectively while GPx and GR are the important constituents of glutathione metabolism, wherein GR plays a key role in the maintenance of cellular reduced glutathione levels, while GPx utilizes the reduced glutathione and protects the cellular systems against the toxicity of hydrogen peroxide. In the present study, we found a significant reduction in the activity levels of SOD, CAT, GPx and GR with a concomitant increase in the lipid peroxidation levels and hydrogen peroxide content in the testis of STZ

alone treated rats suggesting testicular oxidative stress. Furthermore, reduced activity levels of GPx and GR could be linked to improper glutathione metabolism in the testis of rats subjected to STZ and TTZ alone. The results are in agreement with previous studies [46-48]. On the other hand, a significant reduction in the activity levels of SOD, CAT, GPX and GR accompanied by significant elevation in the hydrogen peroxide content and lipid peroxidation levels in the testis of rats subjected to both TTZ and STZ over STZ alone treated rats could reflect the exacerbation effects of TTZ on STZ-induced testicular oxidative toxicity in rats.

One of the major findings of this study indicated that the fertility efficacy of STZ rats was reduced as compared to controls and the effect being further deteriorated in STZ and TTZ treated rats as compared to STZ treated rats. In this study, more number of mating trials could be an indication of intact sexual behaviour in experimental rats and therefore, this could not be a reason for the deterioration of fertility efficacy. Whereas the diminished selected reproductive endpoints such as cauda epididymal parameters and Leydig cell steroidogenesis might be responsible for compromised fertility efficacy in rats subjected with STZ alone or in combination with TTZ as compared to controls. This notion is supported by earlier studies [49]. Furthermore, the pre-implantation and post-implantation loss in female rats cohabited with STZ alone or in combination with STZ plus TTZ as compared to controls could be an indication of paternal-mediated developmental toxicity. Based on the results (Table 1), we hypothesize that pre- and post-implantation loss in females cohabited with STZ-induced diabetic male rats could be due to enhanced abnormalities at early and late spermatogenesis, while enhanced pre-implantation loss in females cohabited with rats subjected to both STZ and TTZ could be attributed to enhanced abnormalities at early stages of spermatogenesis. Further, studies are in progress in our laboratory towards the assessment of male reproductive health and fertility assays of F1 generation rats delivered to females cohabited with STZ and TTZ rats.

Conclusion

The findings i.e. elevated levels of blood sugar levels in rats subjected to both TTZ and STZ as compared to STZ alone treated rats might be suggestive of a) reduced secretion of insulin from the pancreas, b) damage to the pancreas and a and b. Therefore, consumption of TTZ might be additive factor to aggravate diabetes condition in humans and thus negatively target many organ systems including male reproductive tract and its functions. The findings indicated that the TTZ exhibits synergistic effects on testicular and epididymal functions in STZ induced diabetic rats. The aggravating negative effects on male fertility could be ascribed to reduced testicular steroidogenesis and oxidative damage t the level of Sertoli cells, and Leydig cells STZ induced diabetic rats. The present results caution the excess consumption of TTZ in general and in particular during diabetic conditions.

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Conflicts of interest

There are no conflicts of interest to declare.

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