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Original Article

Reiterate and integrate the adverse effect of antipsychotic agent and its attenuation by Ginkgo Biloba Extract on reproductive system and functions of rodents

Noorah Saleh AL-Sowayan[*1], Aliyah Tariq Sheikh[2]

[1]Department of Biology, Faculty of Science, Qassim University, P. O. Box: (30230), Buraydah (51744), Saudi Arabia.
[2]M. Phil. in Molecular Biololgy, CEMB (Center of Excellence for Molecular Biology), Lahore Pakistan.

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ABSTRACT
The psychotic drug agents are linked and believed to increase risk of sexual dysfunction by increasing prolactin levels. Ginkgo Biloba Extract (GBE) is reported much effected in treating antidepressant induce sexual dysfunction. The current study was carried out in two phases. In first phase of study we did the evaluation of effect of hyperprolactinemia caused by CPZ(Chlorpromazine)on ovarian follicular growth, gonadotrophin and changes in ovarian hormone in adult female rats while in second phase investigation of prophylactic role of GBE against testicular damage, oxidative stress and caudal sperm indices in CPZ treated rodent model was done. In 1st study we divided the animals in 4 groups, each group was consisting of 5 rats.1st group was control while other three groups were treated with different doses of CPZ e. g 5mg/kg/day,15mg/kg/day, and 30mg/kg/day respectively for 2 weeks, CPZ was given via gavage. On 15th day, the animals were sacrificed by decapitated, ovaries were removed and stained with H&E and later their histomorphometric examination was done. The serum levels of LH (Luteinizing hormone), FSH (Follicle Stimulating Hormone), Estradiol (E2), Progesterone and Prolactin levels were measured. CPZ treated groups showed varied amount and size of atretic follicle. The Higher rate of dysfunctional ovaries were seen in those groups which were treated with higher doses of CPZ. Similarly, higher concentration of progesterone and prolactin while lower concentration of FSH, E2, LH in sera and decreased rate of pregnancy was also observed in treated groups. The rate of toxicity was directly proportion to quantity of CPZ dose. In 2nd phase of study the animals were divide into 5 groups and each had 5 rats.1st was control,2nd was treated with CPZ alone while other 3 were firstly treated with single dose of CPZ (30 mg/kg) and then treated with different doses of GBE i-e 45mg/kg,90mg/kg, and 200mg/kg respectively for 20 days. CPZ and GBE were given via gavage. On 21st day, at the end of experiment, the animals were sacrificed by de -capitated using guillotine and then their reproductive organs (testis, epididymis) were removed for further study. Decreased weight of testis and epididymis was also noted in CPZ treated groups. Moreover, degeneration of seminiferous tubules, reduction in sperm motility and count was also observed after CPZ toxicity. In addition to this, increase in the germ cell apoptosis was also noted; the levels of SOD, Testosterones, and CAT got reduced while MDA was increased in treated groups. The groups treated with GBE showed visible and significant improvement in all above said pathological alterations. The results of current study showed that 200mg/kg dose of GBE was more effective in protecting rodents against reproductive toxicity caused by CPZ.
Keywords: Hyperprolactinemia, Antipsychotic agents, Ginkgo Biloba, reducing reactive oxidation species, reproductive organs.

Address for Correspondence: Noorah Saleh AL-Sowayan, Department of Biology, Faculty of Science, Qassim University, P. O. Box: (30230), Buraydah (51744), Saudi Arabia; E-mail: knaaj@yahoo.com

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INTRODUCTION

Chlorpromazine is an anti-psychotic medication in a group of drugs called phenothiazines. It works by changing the actions of chemicals in our brain. Chlorpromazine is used to treat psychotic disorders such as schizophrenia or manic-depression, and severe behavioral problems in children ages 1 through 12 (1,2). Chlorpromazine is also used to treat nausea and vomiting, anxiety before surgery, chronic hiccups, acute intermittent porphyria, and symptoms of tetanus. (3) Common side effects include movement problem, sleepiness, dry mouth, low blood pressure while standing and increased weight (4). All antipsychotic drugs are thought to aggravate cardiac arrhythmia, postural hypotension and sexual dysfunction (5-10). Chlorpromazine is a very effective antagonist of D2 dopamine receptors and similar receptors, such as D3 and D5. Unlike most other drugs of this genre, it also has a high affinity for D1 receptors. Blocking these receptors causes diminished neurotransmitter binding in the forebrain, resulting in many different effects. Dopamine, unable to bind with a receptor, causes a feedback loop that causes dopaminergic neurons to release more dopamine. Therefore, upon first taking the drug, patients will experience an increase in dopaminergic neural activity. Eventually, dopamine production of the neurons will drop substantially and dopamine will be removed from the synaptic cleft. At this point, neural activity decreases greatly; the continual blockade of receptors only compounds this effect (3).

Prolactin is secreted from the pituitary gland in response to eating, mating, estrogen treatment, ovulation and nursing. Prolactin is secreted in pulses in between these events. Prolactin plays an essential role in metabolism, regulation of the immune system and pancreatic development.

Pituitary prolactin secretion is regulated by endocrine neurons in the hypothalamus. Prolactin provides the body with sexual gratification after sexual acts. The hormone counteracts the effect of dopamine, which is linked to sexual arousal. This is thought to cause the sexual refractory period the amount of prolactin can be an indicator for sexual satisfaction and relaxation. Unusually high amounts are suspected to be responsible for impotence and loss of libido.

Elevated levels of prolactin decrease the levels of sex hormones — estrogen in women and testosterone in men, while the effects of mildly elevated levels of prolactin are much more variable, in women, substantially increasing or decreasing estrogen levels. (11). Hyperprolactinemia or hyperprolactinemia is the presence of abnormally high levels of prolactin in the blood In women, a high blood level of prolactin often causes hypoestrogenism with anovulatory infertility and a decrease in menstruation (35,36), while in men, the most common symptoms of hyperprolactinemia are decreased libido, sexual dysfunction (in both men and women), erectile dysfunction, infertility, and gynecomastia (8,42,46,47). Its synthesis and release are under the control of peptides, steroids and neurotransmitters. The main inhibitory regulation is made by dopamine, which binds dopamine receptors D2 on the membrane of lactotroph cells. Antipsychotic-drugs block these receptors and thus remove the inhibitory effect of dopamine on prolactin secretion. Clinical and endocrinological changes of hypogonadism also likely occur during chronic antipsychotic induced hyperprolactinemia because hyperprolactinemia may cause clinically significant side effects in patients treated with antipsychotic medications, therefore, clinicians should be familiar with the evaluation and treatment of antipsychotic-induced hyperprolactinemia (11-14).Previous researches suggests that SSRIs cause dysfunction in all phases of the sexual response cycle, predominantly in the phases of desire and orgasm in males and arousal in females (15-20). TCAs have been found to have effects on desire and orgasm, although they may also affect other phases of the sexual cycle since they modify several neurotransmitters (21). SD (sexual dysfunction) is known to decrease compliance with antidepressant treatment, and effective strategies for its management are therefore essential (22,23,21).

Pharmacogenetic research has only recently begun to identify genetic markers that may predict response to antidepressant treatment (24-29). These markers may facilitate the prediction of adverse events such as SD during antidepressant treatment (30). In a study of the treatment of premature ejaculation, long allele homozygotes showed a better SSRI treatment response than short allele carriers (31-34). The major effects of hyperprolactinemia in women are amenorrhea, cessation of normal cyclic ovarian function, loss of libido, occasional hirsutism, and increased long-term risk of osteoporosis. The effects in men are impotence, loss of libido, and hypo spermatogenesis (35). Sexual behavior is mainly controlled by a well-organized neural circuit that connects a variety of brain areas, but the activation of this circuit is highly dependent on gonadal hormones (36,37). Subsequent studies showed that hyperprolactinemia impairs male sexual behavior in rats and mice (38-42).

Although hyperprolactinemia is commonly associated with T deficiency in men and with

ovarian dysfunction in women, it suppresses sexual desire without causing obvious gonadal steroid deficiency (43). All antipsychotic-drugs block D2 receptors and all can induce hyperprolactinemia. Nonetheless, it seems that the faster the antipsychotic-drug dissociates from D2 receptors, the lesser the increase of prolactin in the plasma. (44,45). The role of their metabolites should also be considered. For these reasons, one can distinguish prolactin-raising and prolactin-sparing antipsychotics. Hyperprolactinemia inhibits the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (by increasing the release of dopamine from the arcuate nucleus, which in turn inhibits the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland and results in diminished gonadal sex hormone production (35,46). On the other hand, physiologic function of follicular growth and granulosa cells mainly depend on serum levels of FSH and LH. Therefore, the dysregulation of hormones in which their source is ovarian, will lead to important problems in fertilizing potential (47,48). This disorder in the function of gonadotropins is related to the pituitary gland and its feedback mechanisms. since prolactin is antagonized by dopamine and the body depends on the two being in balance, the risk of prolactin stimulation is generally present with all drugs that deplete dopamine, either directly or as a rebound effect (11).

Ginkgo biloba Extract(GBE)is derived from the leaf of the Chinese ginkgo tree and noted for its cerebral enhancing effects, was found to be 84% effective in treating antidepressant-induced sexual dysfunction predominately caused by selective serotonin reuptake inhibitors (49). The study of Kang Bj, Lee Sj and Kim MD (50); did not replicate a prior positive finding supporting the use of Ginkgo Biloba Extract(GBE) for antidepressant, especially SSRI, induced sexual dysfunction (SD). "Ginkgo Biloba Linne", is a tree belongs to family Ginkgoaceae. It is thought to have been preserved by priests in China and Japan who cultivated it on temple grounds (61-63). The tree has a long medicinal history being recorded as early as 2800 BC in the Chinese literature (63). GBE has been used in the treatment of cerebrovascular and peripheral vascular disorder s (64). In addition, ginkgolides A, B, C and M have been shown to check the platelet activating factor thereby preventing the bronchoconstriction, hypotension, cutaneous vasodilatation and finally the release of inflammatory compounds (64). GBE has antioxidant and hepatoprotective effects and can inhibit liver fibrosis in rat of non-alcoholic steatohepatitis and carbon tetra chloride (65-67). A previous study, reported that repeated intake of GBE enhanced cell proliferation and neuroblasts differentiation in the mouse hippocampal dentate gyrus and consumption of GBE may be helpful to increase endogenous neurogenesis in adults (68-70). GBE facilitates microvascular circulation that may physiologically lead to an improvement in sexual function in animal studies (71). There is evidence that ginkgo extract may directly elucidate smooth muscle relaxation, likely via effects on the NO pathway. The extract has an effect in human and rabbit corpus cavernosum tissue using organ bath and electric field stimulation (72). An open label clinical trial used ginkgo extract to treat arterial erectile dysfunction. Sixty patients who had not improved with papaverine injections of up to 50 mg were treated with 60 mg/day ginkgo extract for 12 to 18 months. The penile blood flow was re-evaluated by duplex sonography every 4 weeks. Fifty percent of patients had regained potency (73). Another open label trial showed that ginkgo is effective in treating antidepressant-induced sexual dysfunction. Ginkgo generally had a positive effect on all four phases of the response cycle (i.e. desire, excitement, erection and lubrication), orgasm and resolution (74). Furthermore, by stopping NF-kB and then iNOS by using antioxidants has already proved to be effective in attenuating the CIS-induced testicular injury (75-79). It is therefore, one of the objective of the present study is to elucidate the association between antipsychotic agent, SD and infertility and then to explore the ameliorating effect of GBE on reproductive organ toxicity caused by CPZ. Moreover, it is reported that Cyclooxygenase- (COX)-2 plays a physiological role in inflammation and tumor proliferation and its selective inhibitors have been found to be effective in ameliorating CIS-induced nephrotoxicity in rats (80,81).

MATERIALS AND METHODS

Animals: We conducted this study in two phases. In 1st phase, we took 40 adult female Wister rats. Their weight was 160-180 gm and they were 70 days old, while in the 2nd phase of study we took 40 adult male Wister rats, and their weight was 120 - 240 grams. We obtain animals from" Animal house Alqaseem University", Buraidah, Saudi Arabia. The animals were acclimatized in an environment with temperature of 22-24 ^{0}C and 12-hour light and 12-hour dark schedule. This study was approved by "Animal Ethics Committee Alqaseem University Buraidah".

Chemical/Drugs/Plant: CPZ was bought from Sigma-Aldrich Company Germany. In phase 1 of current study, CPZ was used at 3 dose levels in 3 different groups, which was 3mg/kg, 10mg/kg, 30mg/kg respectively, based on a previous study (48).

The drug was dissolved in 0.5% methyl cellulose solution (51), while in the phase 2, GBE was obtained from General Nutrition Corporation, USA. Anti-Rat Primary antibodies (1:100 dilution) for COX 2 (Clone SP-21) and NF-kBP^{65} (Rel A, Ab-1) from rabbit and Polyvalent biotin-labelled goat anti-rabbit secondary antibody (1:200 dilution) were taken from ThermoFisher Scientific USA. Moreover, other chemicals like Thiobarbituric acid, 1,1,3,3-Tetramethoxy-Propane, Sulphuric acid, Phosphoric acid and Hydrogen Peroxide was brought from Sigma-Aldrich Company Germany. The GBE was also standardized to Ginkgo Flavonoglycosides(24%) & Terpene Lactones (6%). The CPZ was administer to rats by oral gavage. Tissue images were taken by Optical Microscopy (Olympus DP71).

Experimental Protocols

In phase 1 of current study, the female rats were divided into 4 groups and each group had 8 rats in it, while in phase 2 the male rats were divided into 5 groups and each group had 10 rats.

In phase 1, the control group was given 5ml/kg of 0.5% methyl cellulose solution once in a day for 14 days continuously. To other 3 groups the CPZ was given at 3 different dose level which were 3mg/kg/day, 10mg/kg/day, 30mg/kg/day respectively for 14 consecutive days.

One week before the end of treatment, 4 female Wister rats from all groups were chosen and placed with the sexually active Wister rats for mating. After 21-23 days, the neonates were counted. At the end of drug treatment i-e on 15th day, a few of animal from each group was taken and killed by rapid decapitation and blood samples were collected from juggler vein. Later the serum was harvested and frozen for further studies.

The ovaries were surgically removed and fixed in formaldehyde acetic solution till further study.

The samples were processed through paraffin embedding and were cut with microtome, stained with hematoxylin and eosin. The follicles in ovarian sections were characterized per their sizes i-e 100, 101-200, 201-300, 301-400, 401-500 and larger than 500µm.

Follicles with complete layer of flattened granulosa cells, normal nucleus and oocyte with normal cytoplasm were considered normal follicles.

While those with pyknotic nucleus and any other kind of cytoplasmic damage were considered abnormal follicles.

Follicular number was estimated by counting follicles in all slides (52). We also counted corpora lutea number per ovary. The blood serum was harvested and frozen till further investigation.

Radio immunoassays of prolactin, LH, and FSH in sera were done. 100µl of sera was added to 100µl of hormones labeled with rabbit antisera in 0.01M phosphate buffer. Anti-rat prolactin, LH and FSH was diluted to 1:5000, 1:10000 and 1:2500 respectively.

Goat anti-rabbit IgG at a dilution of 1:10 (200µl was added to the mixture and mixture was kept at 40^0C for 18 hours, then it was centrifuged at 2000xg for 30 minutes then we measured radioactivity level from the pellet by a gamma counter.

Radio immunoassay of serum estradiol and progesterone was done per manufacturer's instructions. We measure the concentration of serum estradiol by CPZ kits (Cisbio-Bioassay France). The radioactivity was measured in resultant pellet (53).

In phase 2nd of current study, we divided male rats in 5 group and each group has 10 rats. The control (group 1) was injected with 5ml of saline water/kg body weight intraperitoneally for 20 days. Group 2 was given CPZ alone as single dose of 30mg/kg; I.P on first day of treatment, to induce testicular toxicity in rats (82).

Group 3,4,5 was injected with a single dose of CPZ 30mg/kg of body weight on first day of treatment and after I hour different doses of GBE were injected to other 3 groups for 20 days, which are as follows:

Group 3 was given GBE 45mg/kg of body weight/day. Group 4 was given GBE 90mg/kg of body weight/day. Group 5 was given GBE 200mg/kg of body weight/day for 2o days.

Doses of GBE were selected on bases of a previous study (83). After 20 days of treatment, rats were killed and weighted. Later, their testes and epididymis were dissected and weighted too.

Total no of sperms was counted by Neubauer Hemocytometer. Cauda epididymis was minced in 5ml saline and then incubated at 37^{o}C for 30 min. The sperms came out of epidydimal tubules. The percentage of motile sperms were counted by phase contrast microscope with power 400X. Total no of sperm/gm of right side of cauda was calculated.

Biochemistry: Homogenization of testes was done in ice cold KCl buffer (160mmol/L). we prepared aliquots of it. Supernatant was safe for lipid per oxidation, CAT and other Biochemical markers. In testicular tissues the activity of super oxide(SOD) and myeloperoxidase(MPO) were determined as described in (84,85) and (86) respectively. And total Prolactin was estimated by Lowry's method.

Histology: small pieces of testis were fixed in 10% neutral phosphate buffered formalin and then after getting stained with H&E ,5µm thick section were examined under Leica DMRB/E microscope. Apoptosis were determined by using "TUNEL Technique "and for that we used A pop Taq plus peroxidase Insitu Apoptosis detection kit. It was done per manufacturer's instructions. Sequential mounted sections of specimen were used for further immunohistochemical analysis. These sections were incubated in primary antibodies overnight at 4^0c.The anti-rat primary antibodies were taken from Thermo Fisher Scientific and whole procedure was done as per instructions given within kit. Next day the slides were washed with PBS and second time incubation of tissues section was done with polyvalent biotin labelled goat anti-rabbit secondary antibody (1:200 dilution) for 10 minutes. Staining of sections were done by Universal LSAB plus Kit & a DAB plus substrate kit was used as a chromogen; lastly the section was stained by H&E. At the end, tissue images were taken by optical microscopy.

Statistical analysis: The data was presented as group mean +- SE. The statistical analysis was done by ANOVA and SPSS version 16 (Chicago, IL, USA). At the end Dennett's' test was done.

RESULTS

Results from study 1:

Biochemistry: The serum levels of prolactin significantly (P<0.05) increased in CPZ treated groups, which was CPZ dose-dependent, while in control group the prolactin level was constant. The serum levels of FSH and LH were significantly decreased(P<0.05) in CPZ treated animals. This decrease was also CPZ dose-dependent. Moreover, the serum levels of estradiol also got significantly (P<0.05) decreased, furthermore, the progesterone level got (P<0.05) increased in CPZ treated groups, which was also dose-dependent. (Table 2).

Fertilizing index: The fertilizing index in the control and test groups was also observed. In CPZ-treated groups, both high doses showed negative fertilizing index because these groups produced no neonates, while the control and the group treated with CPZ at the dose of 3 mg/kg/day showed positive fertilizing indexes (Table 1).

Histology: after doing histological analysis, we found that in CPZ-treated groups, the total number of normal follicles significantly (P<0.05) got decreased as compared to control group. Control group ovaries had shown all types of follicles of different developmental stages from primordial to graafian follicles. Follicles were of different sizes that ranged from <100 µm to >500 µm. But groups treated with high doses of CPZ did not shown any large antral follicles (>500 µm). The numbers of atretic follicles were more in CPZ treated groups as compared to the control group and this was depended on the intensity of the dose of CPZ (Figure1). Comparing the rate of normal follicles between the control and CPZ groups showed a significant (P<0.05) decrease in the CPZ groups, moreover, Higher CL sizes were also seen in the groups treated with higher doses of CPZ. (Tables 2-5).

Results from study 2:

After performing TUNEL technique, the apoptosis in seminiferous tubule was determined. The figure 2 depicted the brown color nuclei in seminiferous tubules form all groups. These are TUNEL positive cells. Different levels of apoptosis levels were seen in different experiment groups. Variable levels of cox-2 expression were also observed in different experimental groups. (figure 3). While CPZ treated group showed high increased level of Cox 2 expression as compared to control and GBE treated groups. Furthermore, variable levels of NF-kb expressions were also noted in different groups. NF-kb levels were higher in CPZ treated groups as compared to control and GBE treated groups. (figure 4)

Histopathology

Cross sections of testes of control shows normal arrangement of sertoli and germ cells, while CPZ treated testes showed significant damage to seminiferous tubules. GBE treated groups were less affected by CPZ; here we saw moderate to severe testicular atrophy with degeneration and cellular disorganization in seminiferous tubules, depletion of leydig cells were also observed between the tubules in CPZ treated group. In GBE treated group, slight to moderate degeneration of seminiferous epithelium was also found. GBE treated group showed normal testicular morphology. CPZ treated group showed significant decrease in weight of testes and epididymis as compared to control group (Table 1). GBE treated group had shown better weight of tests (p<0.05 & p<0.001) as compared to CPZ alone treated group.
Significant decrease (P<0.001) in sperm count and motility was found in CPZ treated groups. While better sperm count and motility were seen in all GBE treated groups (table 1).

Oxidative stress: Significant (P<0.01) increase in activation of MPO and increased level of MDA, while significantly (P<0.001) decrease level of SOD and CAT were observed in CPZ treated groups which showed the potent oxidative action of CPZ on testicular tissues. Treatment with GBE reduced the toxicity and oxidative stress caused by

CPZ and normalizes the abnormalities in MDA, CAT, MPO and SOD levels to much extend. This all proves the protective effect of GBE on CPZ treated groups.

DISCUSSION

Antidepressant therapy, although effective for treating the symptoms of depression, frequently induces or exacerbates the relatively common side effect of sexual dysfunction, which occurs in approximately one half of patients. These sexual side effects may affect the patients' quality of life and can lead to non-compliance and relapse in long term treatment. Although many studies of antidepressant-induced sexual dysfunction have been conducted in Western society, there is little information on incidences of such in Saudi Arabia. In the 1st phase of present study, we tried to explore the dose dependent adverse effects of an antipsychotic drug(CPZ) on structure and function of female rat's reproductive. In this study, we came across with some interesting biochemical observations and that is, the serum levels of prolactin increased in CPZ treated groups, which was CPZ dose-dependent, higher the dose of CPZ, higher was the prolactin level in serum. While in control group the prolactin, level was constant. The serum levels of FSH and LH were also decreased in CPZ treated animals. This decrease was also CPZ dose-dependent. Moreover, the serum levels of estradiol also got decreased, in contrast to that, the progesterone level got increased in CPZ treated groups, which was also dose-dependent. As we know from previous studies that Elevated levels of prolactin decrease the levels of sex hormones estrogen in women and testosterone in men, while the effects of mildly elevated levels of prolactin are much more variable, in women, substantially increasing or decreasing estrogen levels. (11), our results were well in accordance with this study.

Another study also proved that in women, a high blood level of prolactin often causes hypoestrogenism with anovulatory infertility and a decrease in menstruation (35,36). We know from previous researches that dopamine plays a crucial role in tonic inhibition of prolactin secretion (54,) and it acts on lactotroph cells in the anterior pituitary gland and stops the secretion of prolactin (56). Same results were shown from another study that Chlorpromazine is a very effective antagonist of D2 dopamine receptors and similar receptors, such as D3 and D5**,** hence it stops the production of dopamine secretion and thus the prolactin level gets increased in serum (11,55). Hyperprolactinemia inhibits the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus (by increasing the release of dopamine from the arcuate nucleus, which in turn inhibits the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland and results in diminished gonadal sex hormone production (35,46). Results of current study agreed with (11,55) because histological findings showed that in CPZ-treated groups, the total number of normal follicles got decreased as compared to control group, moreover, groups treated with high doses of CPZ did not shown any large antral follicles. Other than that, the numbers of atretic follicles were more in CPZ treated groups as compared to, the control group and this was depended on the intensity of the dose of CPZ. Comparing the rate of normal follicles between the control and CPZ groups showed decrease in the CPZ groups, along with that, Higher CL sizes were also seen in the groups treated with higher doses of CPZ. Same is reported in other studies that high prolactin levels stop the secretion of GnRH from the hypothalamus axis and then can prevent luteolysis and thus increased the numbers of persisting CL (57-59). The pulsatile secretion pattern of GnRH induces the cyclic release of LH and FSH. From a previous study, it is reported that inhibition of GnRH results in reduced LH and FSH levels (60-63). Physiologic function of follicular growth and granulosa cells mainly depend on serum levels of FSH and LH, therefore, the dysregulation of hormones in which their source is ovarian, will lead to important problems in fertilizing potential (47,48,). In current study, we found that the serum level of estrogen was decreased while progesterone increased in CPZ induced groups; higher the dose of CPZ, higher will be the concentration of progesterone and in the same manner, higher the dose of CPZ that much lower will be the concentration of E2. Hence, the serum level estrogen was inversely proportional to the dose of CPZ and serum level of Progesterone was directly proportion to the dose of CPZ. And it showed that increased levels of prolactin with a simultaneous effect of progesterone resulted in remarkable follicular atresia (62), this finding also showed that the observed CLs were considerably active. Due to increased progesterone levels and absence of appropriate feedback for androgens and E2 secretion and to restart a new cycle follicular growth depression happened in the ovaries of CPZ induced groups (64,65). Decrease in E2 levels in CPZ treated groups in our study, resulted in decrease in number of primordial follicles which is in accordance with (66,67). Hence we can say that CPZ is directly or indirectly involved in causing hyperprolactinemia and in blocking of hypothalamus pituitary axis, which thus, stops gonadotropin secretions, therefore, our results showed less potential fertility in CPZ treated groups at high doses.

2nd phase of study: The second phase of the present study explored different levels of apoptotic changes in CPZ treated animals as they showed high levels of Cox-2 expression and NF-Kb expression as compared to GBE protected groups. NF-KB function as a link between oxidative damage and inflammation. This factor transduces oxidative stimuli to nucleus to modulate the expression of many genes involved in the inflammatory responses e-g iNOS (143,144). It is believed that increase in amount on iNOS caused increase in production of Nitrogen, which in turn causes cytotoxic effects and induced germ cell apoptosis (78, 145,146). CPZ treated groups showed significant damage to the seminiferous tubules as compared to control and GBE protected groups. Significant decrease in sperm count, and motility was seen in CPZ treated groups as compared to GBE induced groups. Hence, pretreatment with GBE showed ameliorating effect against the histopathological lesions caused by CPZ, along with that the apoptotic changes caused by CPZ treatment was also reduced in GBE protected groups. Higher the dose of GBE, higher was the protective effect on GBE treated groups. Hence, level of MPO, CAT and SOD in CPZ treated groups showed normalization after being treated with GBE. Inhibiting NF-kb by using anti-oxidations are very effective in attenuating CPZ induced testicular injury (75-78). Cox-2 selective inhibitors have been found to be very effective in ameliorating CPZ induced nephrotoxicity in rates (80,81). Our study showed that after treatment with GBE the CPZ treated groups showed normal to moderate levels of Cox-2 expression and NF-kb expression. These results agree with previous study which says that protective effect of herbal plants against reproductive damage (caused by CPZ), is due to the presence of antioxidative properties of herbal plant (87). Our current study, is in accordance with another study which says that Ginkgo biloba extracts shows potent antioxidant activity and are capable, *in vitro*, of scavenging various reactive oxygen species (88-93). In another study, it is showed that GBE can inhibit or reduce the functional and morphological impairments observed after lipoperoxide release (94-96). Extracts from the leaves of *Ginkgo biloba* are reported to be effective at increasing vascular relaxation via a nitric oxide pathway. (97-99). Another study showed that Ginkgo extracts (specifically the bilobalide component) can suppress hypoxia-induced membrane breakdown in the brain (100). Experimental evidence indicates Ginkgo's effect on the central adrenergic system might also be involved in its therapeutic actions, since the extract appears to reactivate noradrenergic activity, particularly in aged animals (101-104). The anti-stress and neuroprotective effects of *Ginkgo biloba* extract might also be related to its effect on glucocorticoid biosynthesis. Ginkgo extract – and specifically its components ginkgolide A and B – decreases corticosteroid synthesis (105,106). Ginkgo extract has been used successfully to treat impotence and sexual dysfunction secondary to antidepressant medication use (107-109), and our results are in accordance with this study.

The obtained results agree with (110,111) who reported that although the human body continuously produces free radicals, it possesses several defense systems, which are constitutes of enzymes and radical scavengers such as superoxide dismutase, catalase and glutathione peroxidase while non-enzymatic category contains vitamin C, E, A, β-carotenoids, uric acid and ubiquinone. These are called "first line antioxidant defense system" but are not completely efficient because almost all components of living bodies, tissues and cells undergo free radical destruction. Spermatozoa, like any other aerobic cell, are constantly facing the "oxygen-paradox"; the excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa and containing leukocytes has been defined as one of the few etiologies for male infertility (112). There are two main mechanisms by which reactive oxygen species (ROS) cause infertility. First, ROS damage the sperm membrane, which in turn, reduces sperm motility and their ability to fuse with the oocyte. Second, ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo (113).

Our results showed that GBE activated the antioxidant enzymes together with the substances that are capable of either reducing reactive oxygen species or preventing their formation, thereby forms some protective mechanisms, which maintain the lowest possible levels of reactive oxygen species inside the cell. Our results indicate that GBE treated groups, have elevated levels of catalase and SOD activity as compared to CPZ treated groups.

Our results agree with (114) who said that GBE can inhibit membrane lipid peroxidation by its antioxidant activity. The present results indicate the decrease in NO by GBE, which agrees with (115) Aqueous and ethanoic extract of roots, flower, and leaves were used in hepatoprotective activity (116,117).Hence Antioxidants are molecules that are capable of slowing or preventing the oxidation of other molecules, thereby protecting cells from damages caused by exposure to free radicals, including reactive oxygen species, which are produced during oxidation reactions in biological cells, antioxidants can be either phytochemicals or vitamins and other nutrients, they range from micro

molecules such as glutathione, vitamins, to macromolecules such as catalase, glutathione and peroxidase (118,119).

Our current study showed many histopathological changes in CPZ treated groups, we found that height of epithelial lining of the seminiferous tubules got reduced, moreover, degenerative change in the spermatogenesis and Leydig cells was also found. Another research study showed that increased morphological defects and production of abnormal sperms may be because of direct toxicity of CPZ, because cellular DNA is a primary target of CPZ in its anti-neoplastic and toxic activity (120-123,126).

Our study showed decrease in serum levels of LH and testosterone in CPZ treated groups, which agrees with the previous study which reported that reduced expression of key enzymatic and non-enzymatic antioxidants in Leydig cells resulted in excessive oxidative stress and enhanced oxidative damage in these cells that resulted in a decline in testosterone secretion (124,125). Our present study reported significant reduction in CAT and SOD and increase in MDA levels in testes of CPZ treated groups and same results were reported by another study in which the Oxidative damage was also seen in testes of CPZ treated rats (126).

Moreover, a previous research reported that GBE treatment effectively alleviated all histological abnormalities induced by doxorubicin in testicular tissue of rats and protected from cadmium chloride and cisplatin induced histological alterations respectively (127-132). And this report is in accordance with our present results in which we found sperm count got increased and sperm abnormalities got decrease in GBE treated groups because of antioxidant potential of GBE. In the present work, significant increase in serum LH and testosterone levels in groups treated with both CPZ and GBE was observed. Furthermore, a previous study showed that GBE limited lipid peroxidation and scavenge lipid radicals both *in vitro* and *in vivo* and actively protected microsomal membranes from oxidative damage (133-136).

The Flavonoids and terpenoids which are the main constituents of GBE are believed to scavenge free radicals and reduce levels of reactive oxygen species (137). Hence, our results showed different levels of apoptotic changes in CPZ treated animals as they showed high levels of Cox-2 expression and NF-Kb expression as compared to GBE protected groups. CPZ treated groups showed significant damage to the seminiferous tubules and decrease in sperm count, and motility. While, pretreatment with GBE showed ameliorating effect against the testicular toxicity caused by CPZ treatment in GBE protected groups. Higher the dose of GBE, higher was the protective effect on GBE treated groups

Conclusion

Depression and antidepressant therapy have been associated with sexual dysfunction. Studies report wide discrepancies regarding frequency, gender, and quality of sexual dysfunction. Antidote strategies are often used to lessen the sexual difficulty associated with these agents. A variety of medications are reported to reverse antipsychotic-induced sexual dysfunction. The ginkgo biloba has been used to treat antidepressant-induced sexual dysfunction. Our result from phase 1 of current study showed hyperprolactinemia in serum of CPZ treated groups and thus, the reproductive hormonal levels were affected badly, which in turn had adverse effect on follicular growth along with that the fertilizing index was decreased too. These adverse effects were dose dependent, higher the dose of CPZ, higher was the adverse effect. In addition to this, our results from the second phase of current study explored that the testicular toxicity caused by CPZ could be protected by GBE pretreatment. In other words, GBE pretreatment can reverse the testicular toxicity in CPZ treated groups; and this is dose dependent, higher the dose of GBE, higher will be the protective effect. CPZ treated groups showed significant damage to the seminiferous tubules as compared to control and GBE protected groups. Significant decrease in sperm count, and motility was seen in CPZ treated groups as compared to GBE induced groups. Hence, pretreatment with GBE showed ameliorating effect against the his

Future directions

Antidepressant-induced sexual dysfunction (SD)becomes an important issue in the context of treatment effectiveness, as antidepressant medications are helpful only insofar as patients take them. Intolerable side effects may be one reason that patients are noncompliant with antidepressant treatment. Given the importance of continuation and maintenance treatment for major depression, researchers are devoting increasing attention to understanding which treatments may be helpful or, alternatively, unhelpful with respect to sexual functioning so that compliance may be maintained and treatment optimized. Management of SD by herbal remedies is useful because of long cultural history of utilization and the current renewed interest in natural products to sustain health globally. As a way recognizing the values and roles of traditional medical knowledge in health care provision, further research into the efficacy and safety of herbal approach for the management of SD is necessitated worldwide. The search for natural supplement from medicinal

plants is being intensified probably because of its reduced side effects, its ready availability and reduced cost. The potency of the herbal plant drug is significant. Therefore, the increasing search for medicinal plants with aphrodisiac potentials has necessitated the need for screening medicinal plants with aphrodisiac potentials. The herbs can be an effective aphrodisiac, moreover, isolation and identification of active constituents from plants may bring a dynamic change in the modern world.

Tables and figures from study 1 and 2

Study 1:

Table 1: Data showing number of neonates and fertilizing index in different experimental groups.

Parameters	Group1	Group2	Group3	Group4
Number of animals examined	3	3	3	3
Mated animals (n)	3	3	0	0
Fertility index (%) 1		100	75	0
Neonates	35	21	0	0

1; Fertility index (%)= (number of pregnant animals/number of animals that copulated) ×100 and CPZ; Chlorpromazine. control (group1), CPZ 3 mg/kg(group2), CPZ 10mg/kgGroup3), CPZ 30 mg/kg(group4).

Table 2: Data showing serum levels of, progesterone and estradiol, LH, FSH and Prolactin in different experimental groups

Hormones	Group1	Group2	Group3	Group4
Prolactin (ng/ml)	53.65 ± 3.06	100.25 ± 13.37	220.75 ± 26.35 $^{a, b}$	239.50 ± 25.$^{82\ a, b, c}$
LH (ng/ml)	0.53 ± 0.05	0.55 ± 0.06	0.25± 0.02 ^{a,b}	0.20 ± 0.02 ^{a,b}
FSH (ng/ml)	3.2 ± 0.48	1.95 ± 0.44	1.33 ± 0.27 ^{a,b}	1.15 ± 0.06 ^{a,b}
E (pg/ml)	40.50 ± 2.62	27.00 ± 1.47	26.20 ± 2.59 ^{a,b}	24.10 ± 0.40 ^{a,b}
Progesterone (ng/ml)	18.10 ± 2.55	21.57 ± 3.11	31.070 ± 3.75 ^{a,b}	34.92 ± 3.71 ^{a,b}

. $^{a, b, c}$; Indicate significant differences (P<0.05) between data of chlorpromazine (CPZ) groups with other groups: control (group1), CPZ3 mg/kg(group2), CPZ10mg/kgGroup3), CPZ 30 mg/kg(group4), respectively. All data are mean ± SD.

LH; Luteinizing hormone, FSH; Follicle-stimulating hormone and E2; Estradiol.

Table 3: Data showing mean no. of normal and atretic follicles on ovaries of different experimental groups.

Parameters (n)	Group1	Group2	Group3	Group 4
Primordial follicles	280.50 ± 11.90	248.70 ± 13.47	127.50 ± 4.19 $^{a, b}$	123.75 ± 9.46 $^{a, b, c}$
Primary follicles	4.65 ± 0.48	3.73 ± 0.48	5.10 ± 0.57	6.50 ± 0.64
Secondary follicles	8.00 ± 0.70	5.50 ± 0.28	5.10 ± 0.28 $^{a, b}$	4.50 ± 0.62 $^{a, b}$
Tertiary follicles	6.40 ± 0.25	5.50 ± 0.28	5.25 ± 0.47	5.05 ± 0.75
Graafian follicles	8.15 ± 0.75	6.90 ± 0.40	5.20 ± 0.28 $^{a, b}$	4.25 ± 0.85 $^{a, b, c}$
Atretic follicles	1.20 ± 0.25	3.50 ± 0.41	12.20 ± 0.29 $^{a, b}$	12.75 ± 0.64 $^{a, b, c}$
Preantral atretic follicles	0.26 ± 0.25	1.35 ± 0.25	6.05 ± 0.48 $^{a, b}$	7800 ± 0.41 $^{a, b, c}$
Antral atretic follicles	1.00 ± 0.00	1.65 ± 0.29	5.20 ± 0.57 $^{a, b}$	5.55 ± 0.70 $^{a, b, c}$
Corpora lutea	10.45 ± 0.28	10.70 ± 0.62	11.00 ± 0.57 $^{a, b}$	11.50 ± 0.64 $^{a, b}$

. $^{a, b, c}$; Indicate significant differences (P<0.05) between data of chlorpromazine (CPZ) groups with other groups: control (group1), CPZ3 mg/kg(group2), CPZ10mg/kgGroup3), CPZ 30 mg/kg(group4), respectively. All data are mean ± SD.

Table 4: Different follicular sizes in ovaries of different experimental groups

Follicles (µm)	group1	group2	group3	group4
<100	290.00 ± 10.97	250.25 ± 13.82	145.00 ± 4.70 [a, b]	140.75 ± 9.93 [a, b, c]
100-200	5.40 ± 0.86	6.10 ± 0.70	12.75 ± 1.29	13.100 ± 2.27
201-300	6.55 ± 0.22	7.45± 0.64	9.70 ± 0.75	9.8± 0.63
301-400	6.70 ± 0.47	6.90 ± 0.70	8.25 ± 0.86	8.3.00 ± 0.12
401-500	2.40 ± 0.44	3.20 ± 0.58	3.28 ± 0.25	3.30 ± 0.86
500<	1.20 ± 0.62	0.70 ± 0.25	0.00 ± 0.00	0.00 ± 0.00

[a, b, c]; Indicate significant differences (P<0.05) between data of chlorpromazine (CPZ) groups with other groups: control (group1), CPZ3 mg/kg(group2) ,CPZ10mg/kgGroup3), CPZ 30 mg/kg(group4), respectively. All data are mean ± SD.

Study 2:

Table 1: The effect of the GBE and CPZ on testicular epididymal weights and on epididymal sperm count, motility, and abnormality in different groups of experiment.

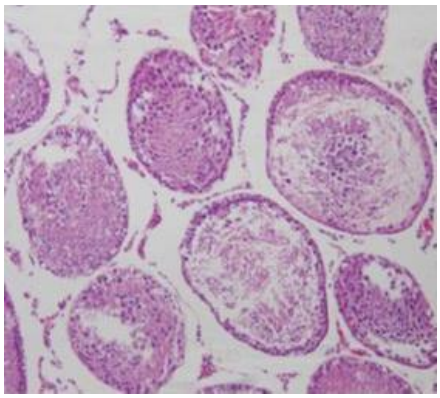
Parameters	Group1	Group2	Group3	Group4	Group5
Testes weight (gm)	3.31 ± 0.06	2.95 ± 0.12*	3.21± 0.08#	3.42 ± 0.18#	3.55 ± 0.13###
Epididymis weight (gm)	1.60 ± 0.06	1.20 ± 0.04**	1.28 ± 0.05**	1.36 ± 0.08	1.34 ± 0.05
Sperm (count.106)/gm of cauda	125.0 ± 7.73	73.94 ± 8.03*	84.40 ± 20.25	84.40 ± 7.77	116.78 ± 8.78
Sperm motility (%)	65.125 ± 3.34	18.25±1.52***	42.2±5.59***###	42.2±3.83***###	52.1 ± 4.2***###

*P < 0.05 versus control, #P < 0.05 versus CPZ, ** P < 0.01 versus control, ###P < 0.001 versus CPZ, *** P < 0.001 versus control.
Group1=control,Group2=CPZ alone 30mg/kg,Group3=GBE 45mg/kg,Group4=Gbe90kg/mg,Group5=GBE200mg/mg

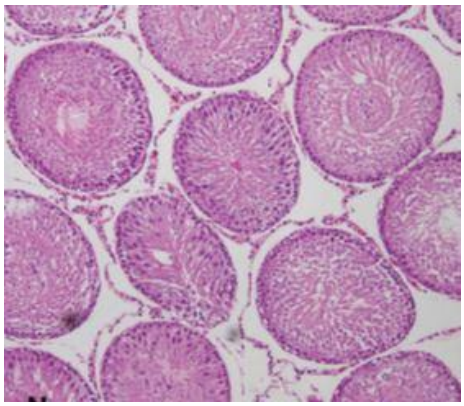
Table 2: Evaluation of MDA, MPO, CAT and SOD in testicular tissue of different experimental groups.

Parameters	Group1	Group2	Group3	Group4	Group5
MDA (nmol/mg protein)	0.95 ± 0.03	1.4± 0.08***	1.0 ± 0.07[c]	0.80 ± 0.06[a]	0.99 ± 0.05[c]
MPO (mu/mg protein)	15.93 ± 0.34	22.65 ± 2.2***	18.00 ± 0.93[b]	18.65 ± 1.54[c]	19.93 ± 0.59[c]
CAT (u/mg protein)	145.59 ± 2.9	80.89 ± 3.24***	122.9 ± 3.9***[a]	124.1±3.64***[a]	134.1 ± 1.88*[a]
SOD (u/mg protein)	3.27 ± 0.02	3.53 ± 0.05***	3.12 ± 0.08[a]	3.21 ± 0.09[a]	3.28 ± 0.06[a]

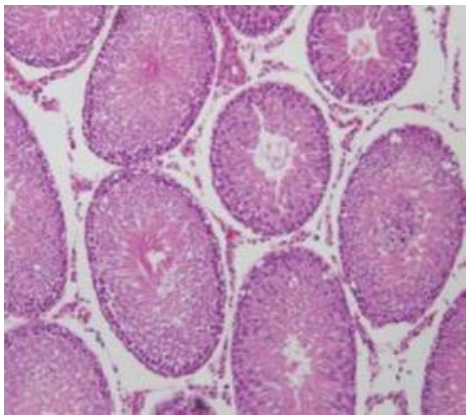
Values are expressed as mean ± SEM of five rats per group. Concentration is expressed as nmol/mg protein for MDA. Activity is expressed as unit/mg protein for CAT and SOD. Activity is expressed as m unit/mg protein for MPO.
t-test: * P < 0.05; *** P < 0.001 versus control. [c]P < 0.05; bP < 0.01; [a]P < 0.001 versus CPZ group.



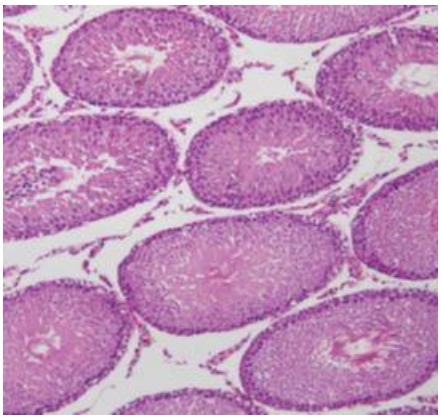
Group 1



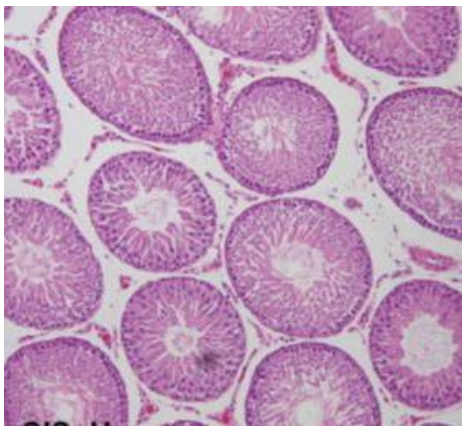
Group 2



Group 3

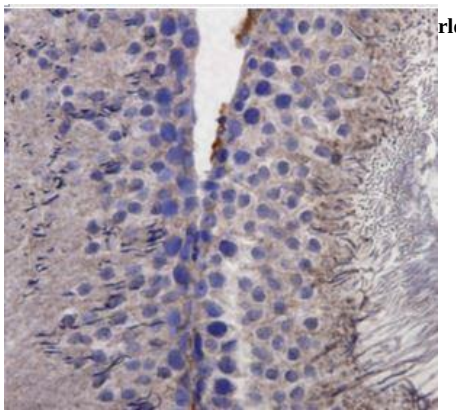


Group 4

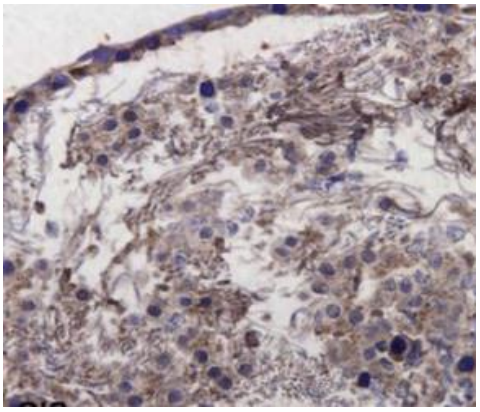


Group 5

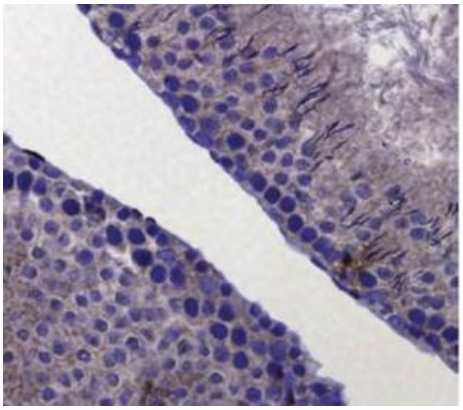
Figure1: Cross section of testis of different experimental group: control (group1), CPZ alone (group2), CPZ+ GBE45mg/kg (group3), CPZ+GBE 90 mg/kg (group4), CPZ+GBE 200 mg/kg (group5). Normal arrangement of germ and Sertoli cells are seen in testes of control group while CPZ treated testes show different levels of damage of seminiferous tubules. GBE treated groups showed much protection against CPZ toxicity and protection is GBE dose dependent. Tissues were counterstained with hematoxylin, 200x.



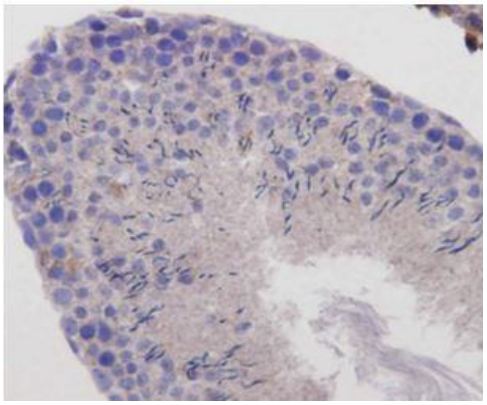
Group 1



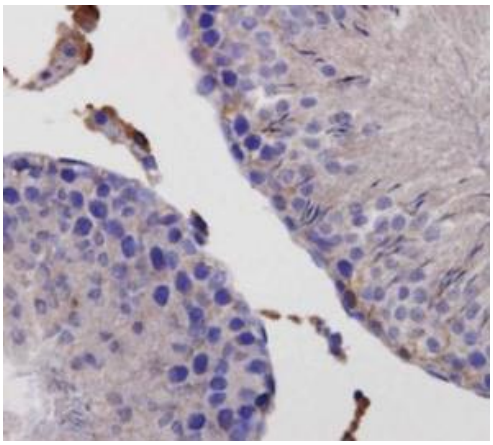
Group 2



Group 3

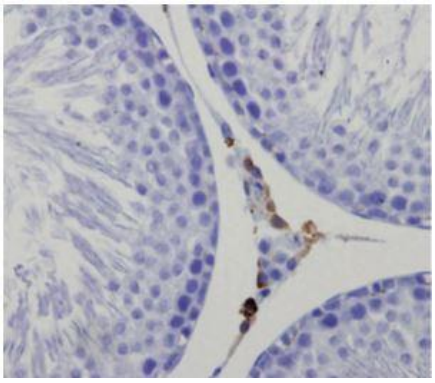


Group 4

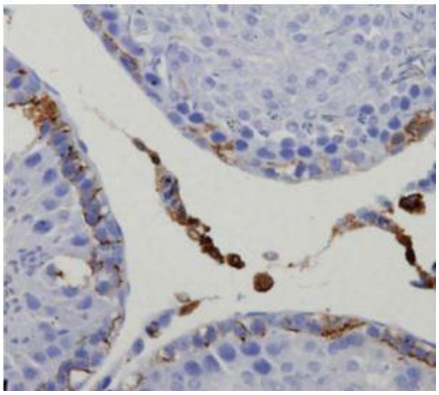


Group 5

Figure 2: TUNEL (Terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling) positive cells in seminiferous tubules of rats in different experimental groups: control (group1), CPZ alone (group2), CPZ+ GBE45mg/kg (group3), CPZ+GBE 90 mg/kg (group4), CPZ+GBE 200 mg/kg (group5). Different levels of apoptosis are seen in different experimental groups. Brown staining shows TUNEL-positive cells. Tissues were counterstained with hematoxylin, 400x.



Group 1



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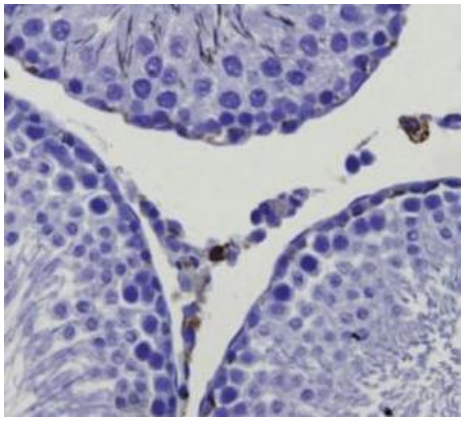

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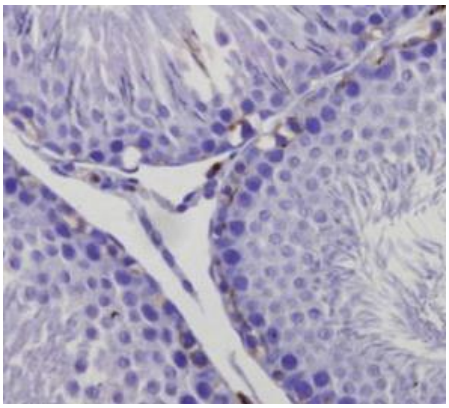
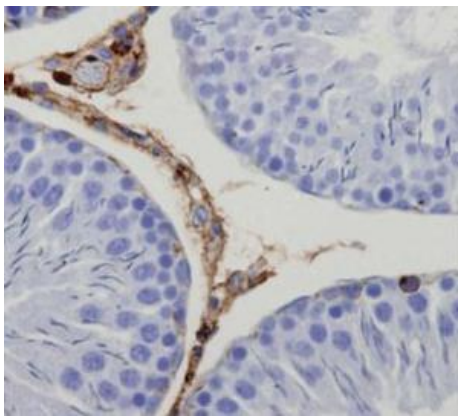
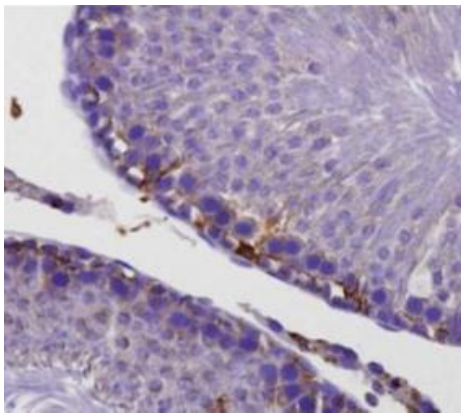


Group 4

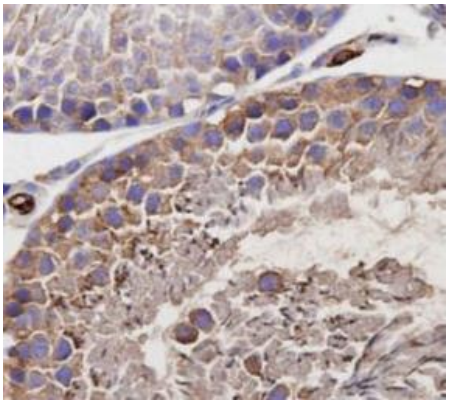


Group 5

Figure 3: Different levels of COX-2 expression are seen by Immunohistochemically in all experimental groups: control (group 1), CPZ alone 30mg/kg (group2), CPZ+GBE 45mg/kg (group3), CPZ+GBE 90 mg/kg (group 4), CPZ+GBE 200 mg/kg (group5). Photomicrographs show variable levels of COX-2 expression in different experimental groups. Brown staining indicates COX-2 expression. CPZ group show increased levels of COX-2 expression compared to N and all three GB-protected groups. Tissues were counterstained with hematoxylin, 400x.



Group 1



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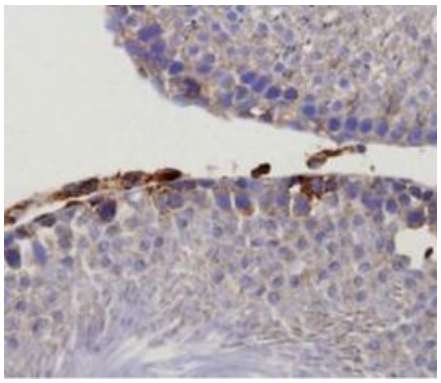
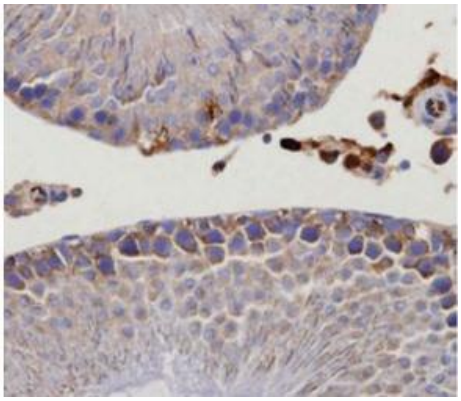
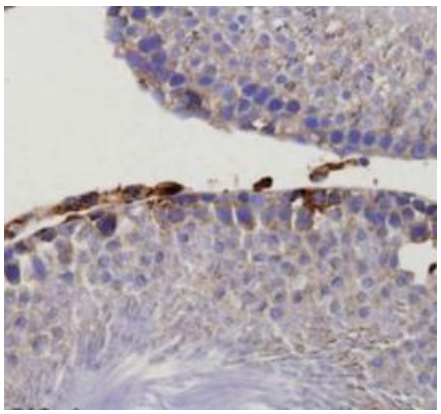


Group 3



Group 4



Group 5

Figure 4: Different levels of NF-kB expression with brown color stained Immunohistochemically is seen in different experimental groups: control (group1), CPZ alone 30mg/kg(group2), CPZ+GBE45 mg/kg (group3), CPZ+GBE90 mg/kg (group4),CPZ+GBE 200 mg/kg (group5). . CPZ-treated rats show increased NF-kB levels as compared to control and all other GBE-protected groups. H&E 400x.

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