Reversible Testicular Toxicity of Piperine on Male Albino Rats

Gopichand Chinta, Mohane Selvaraj Coumar1, Latha Periyasamy2

DBT-Interdisciplinary Program in Life sciences, Pondicherry University, 1Centre for Bioinformatics, Pondicherry University, 2Department of Biochemistry and Molecular Biology, School of Life Sciences, Pondicherry University, Kalapet, Puducherry, India

Submitted: 14-09-2016 Revised: 08-11-2016 Published: 26-07-2017

ABSTRACT
Background: Piperine was widely used in traditional medicine for inducing sterility and abortion. Objective: To evaluate the effect of the piperine on testis of male albino rats. Materials and Methods: Adult male rats were divided into four groups (n = 12). Group I (control): Rats were given vehicle p.o. i.e. 0.5% carboxymethyl cellulose in normal saline daily for 60 days, Group II (ED): Rats received piperine at a dose of 10 mg/kg body weight (b.w.) daily, Group III (E4D): Rats received piperine at a dose of 10 mg/kg b.w. on every 4th day, Group IV (E7D): Rats received piperine at a dose of 10 mg/kg b.w. on every 7th day. Half of the animals from each group were sacrificed after the treatment period (60 days), and the remaining were kept for drug-free withdrawal period (60 days) and then sacrificed. Results: Piperine significantly decreased the reproductive organ weights in groups ED and E4D. Piperine induced hormonal imbalance by altering the serum levels of follicle-stimulating hormone, luteinizing hormone, sex hormone binding globulin, serum, and testicular testosterone in groups ED and E4D. Furthermore, piperine decreased the activity of germ cell markers and Leydig cellular steroidogenic enzymes in the groups ED and E4D after 60 days. All the above-altered values returned to normal levels after withdrawal period. Histopathological findings also supported the above findings. Conclusion: From the above data, it can be concluded that piperine could be a good lead molecule for the development of reversible oral male contraceptive. Key words: Androgen binding protein, germ cell markers, male oral contraceptives, serum hormones, sex hormone binding globulin

SUMMARY
• Piperine was employed for the contraceptive purposes in traditional medicine
• Piperine significantly impaired the spermatogenesis by decreasing the testicular hormone synthesis in groups ED and E4D
• Piperine disrupted the testicular antioxidant system by promoting the ROS production and hydroxyl radical generation in rat testis in groups ED and E4D
• Histopathological evidence supported the disruption of spermatogenesis by piperine

INTRODUCTION
It is estimated that world’s population will reach a staggering mark of 9.1 billion by 2050.[1] Even though sterilization is an effective and permanent means to control population explosion, social structure, health care infrastructure, and other factors prevailing in a particular region make it unsuitable in several circumstances. Alternatively, reversible or temporary contraception such as female oral contraceptives has gained popularity. Majority of men around the world are willing to take a contraceptive pill if such a method is available.[2] Despite this, very few contraceptive methods are available for men as compared to women. None of the hormonal contraceptives such as testosterone, testosterone ethanate, estrogen, anti-androgens, GnRH analogs, and dihydrotestosterone and chemical contraceptives such as depot medroxy progesterone acetate, cyproterone acetate, levonorgestrel, meandomin, α-chlorohydrin, metopirone, gossypol, and serotonin have the features of ideal male contraceptive.[2,3] Developing a reversible oral male contraceptive, which is rapidly effective, devoid of adverse events, does not influence the progeny, is acceptable to both the partners, and is still a dream for the researchers around the world. During the past century, natural products have served as a good source for modern drug discovery. Piperine is one such natural compound isolated from Piper nigrum Linn; Piper longum Linn. belongs to the family of Piperaceae. Piperine is responsible for the pungency of both the herbs.[4] A formulation containing these two herbs as active ingredients was employed for inducing menstruation and as abortificient in Indian traditional medicine.[5] Preclinical studies revealed the effectiveness of piperine in treating ailments such as neuronal difficulties and depression.[6-8] Piperine is known to have antidiabetic,[9,10] anti-inflammatory,[11] antiplatelet,[12] antithyroid,[13] anti-leishmanial,[14] anti-asthmatic,[15] antidiarrheal,[16] and antitumor,[17] and the new creations are licensed under the identical terms. This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

Correspondence:
Dr. Latha Periyasamy, Assistant Professor, Department of Biochemistry and Molecular Biology, School of Life Sciences, Pondicherry University, Kalapet - 605 014, Puducherry, India.
E-mail: lataeperiyasamy6@gmail.com
DOI: 10.4103/pm.pm_405_16

Cite this article as: Chinta G, Coumar MS, Periyasamy L. Reversible testicular toxicity of piperine on male albino rats. Phcog Mag 2017;13:S525-32.
anti-mutagenic,\textsuperscript{[18]} and hepatoprotective activities.\textsuperscript{[19]} Piperine is also known for its bioenhancing properties when simultaneously administered with gallic acid,\textsuperscript{[20]} curcumin,\textsuperscript{[21]} β-carotene,\textsuperscript{[21]} tifenon,\textsuperscript{[22]} and (−) epigallocatechin-3-gallate.\textsuperscript{[23]} It also enhances the bioavailability of clinically valuable drugs such as vascine, sulfadiazine, isoniazid, ethambutol, phenobarbitione, phenyoitin, dapsone, tetracyclines, rifampicin, pyrazinamide, carbamazepine, nimesulide, indomethacin, and ciprofloxacin.\textsuperscript{[24-26]} Based on bioenhancing properties of piperine on antitubercular drugs such as isoniazid and rifampicin, synergistically acting formulations are available for clinical use in India since 2009.\textsuperscript{[16]} A recent survey suggests that piperine is one among the 108 plant active constituents reported to have anti-fertility activity.\textsuperscript{[27]} Furthermore, recent studies have proven the anti-fertility activity of piperine on male albino rats after 30 days of treatment.\textsuperscript{[28-30]} However, the effect of piperine on complete spermatogenic cycle and its withdrawal effects were not investigated. The present study was undertaken to find out the effect of piperine on testes and prostate of male albino rats after the treatment (60 days) and drug-free withdrawal period (120 days) at a dose of 10 mg/kg body weight (b.w.).

**MATERIALS AND METHODS**

**Chemicals**

Piperine of 97% purity was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used for various assays were of analytical grade and were obtained from local commercial sources.

**Animals**

Healthy adult male albino rats of Wistar strain (age: 90 days) weighing 190–210 g procured from the Committee for the Purpose of Care and Supervision of Experimental Animals (CPCSEA) approved vendor (Biogen Laboratory Animal Facility, Bengaluru, Karnataka, India) were used for the present investigation. Animals were housed in polypropylene cages bedded with paddy husk and maintained under well-regulated light and dark (12 h: 12 h) schedule. Rats were fed with standard rat pellet diet (Om Sai Enterprises, Chennai, Tamil Nadu, India) and drinking water ad libitum.

**Maintenance**

All the protocols described in this research work were approved by Institutional Animal Ethical Committee (IAEC No: PU/IAEC/2014/25), Department of Biochemistry and Molecular Biology, Pondicherry University, Puducherry. All the animals were maintained according to the guidelines of CPCSEA, India.

**Treatment**

The dose of the piperine was selected according to the investigations carried out by D'Cruz and Mathur, 2005.\textsuperscript{[29]} Adult male rats were divided into four groups with 12 animals (n = 12) in each group. Group I (control): Rats were given vehicle p.o., i.e., 0.5% carboxy methyl cellulose (CMC) in normal saline daily for 60 days. Group II: Rats were treated with piperine suspended in 0.5% CMC at a dose of 10 mg/kg b.w. p.o. daily for 60 days (group ED). Group III: Rats were treated with piperine suspended in 0.5% CMC at a dose of 10 mg/kg b.w. p.o. on every 4th day for 60 days (group E4D). Group IV: Rats were treated with piperine suspended in 0.5% CMC at a dose of 10 mg/kg b.w. p.o. on every 7th day for 60 days (group E7D). Half of the animals from each group were sacrificed after 60 days of treatment. Remaining animals were kept for the drug-free withdrawal period of another 60 days and then sacrificed (total of 120 days). In this study, treatment period was considered as Phase I and withdrawal period as Phase II.

**Collection of serum**

Blood samples were collected from control and treated groups after treatment and withdrawal periods from the retro-orbital plexus. Blood was centrifuged at 1500 ×g for 15 min. Serum was separated and stored at −20°C in microfuge tubes until use.

**Organ weights**

At the end of the treatment (60 days) and withdrawal periods (120 days), the testes and ventral prostate were dissected out and weighted.

**Testes index, testicular coefficient, and gonadosomatic index**

Testis index was calculated by dividing the left testis weight with the total b.w. and multiplying with 100.\textsuperscript{[31]} Testicular coefficient was calculated by dividing total organ weight by b.w. and then multiplying it with 100.\textsuperscript{[32]} Gonadosomatic index (GSI) was calculated by dividing gonad weight with total b.w. and then multiplying with 100, where gonad weight = (weight of the right testis + weight of the left testis)/2.\textsuperscript{[33]}

**Determination of serum and testicular hormones**

Hormones such as follicular-stimulating hormone (FSH),\textsuperscript{[34]} luteinizing hormone (LH),\textsuperscript{[34]} testosterone (T),\textsuperscript{[34]} progesterone (P),\textsuperscript{[36]} and sex hormone-binding globulin (SHBG)\textsuperscript{[37]} were assayed using standard methods.

**Preparation of homogenates**

Homogenates of testes were prepared separately in ice-cold normal saline by using glass-Teflon homogenizer. Supernatants obtained after centrifugation were used for the biochemical assays.

**Determination of testicular parameters**

Testicular testosterone, total cholesterol, total acid phosphatase (ACP), alkaline phosphatase (ALP), and prostatic ACP were assayed using standard methods.\textsuperscript{[35,36-40]}

**Isolation of Leydig cells**

Isolation of Leydig cells was carried out by using methods reported by Anand \textit{et al.}, with little modifications.\textsuperscript{[41,42]} Animals were sacrificed by cervical dislocation and testes were removed. Decapsulated testes were collected from the retro-orbital plexus. Blood samples were collected from control and treated groups after treatment and withdrawal periods from the retro-orbital plexus. Blood was centrifuged at 1500 ×g for 15 min. Serum was separated and stored at −20°C in microfuge tubes until use.

**Organ weights**

At the end of the treatment (60 days) and withdrawal periods (120 days), the testes and ventral prostate were dissected out and weighted.

**Testes index, testicular coefficient, and gonadosomatic index**

Testis index was calculated by dividing the left testis weight with the total b.w. and multiplying with 100.\textsuperscript{[31]} Testicular coefficient was calculated by dividing total organ weight by b.w. and then multiplying it with 100.\textsuperscript{[32]} Gonadosomatic index (GSI) was calculated by dividing gonad weight with total b.w. and then multiplying with 100, where gonad weight = (weight of the right testis + weight of the left testis)/2.\textsuperscript{[33]}

**Determination of serum and testicular hormones**

Hormones such as follicular-stimulating hormone (FSH),\textsuperscript{[34]} luteinizing hormone (LH),\textsuperscript{[34]} testosterone (T),\textsuperscript{[34]} progesterone (P),\textsuperscript{[36]} and sex hormone-binding globulin (SHBG)\textsuperscript{[37]} were assayed using standard methods.

**Preparation of homogenates**

Homogenates of testes were prepared separately in ice-cold normal saline by using glass-Teflon homogenizer. Supernatants obtained after centrifugation were used for the biochemical assays.

**Determination of testicular parameters**

Testicular testosterone, total cholesterol, total acid phosphatase (ACP), alkaline phosphatase (ALP), and prostatic ACP were assayed using standard methods.\textsuperscript{[35,36-40]}

**Isolation of Leydig cells**

Isolation of Leydig cells was carried out by using methods reported by Anand \textit{et al.}, with little modifications.\textsuperscript{[41,42]} Animals were sacrificed by cervical dislocation and testes were removed. Decapsulated testes were incubated in modified Eagle's medium containing 0.25 mg/ml collagenase and 10% penicillin and streptomycin solution for 15 min. The seminiferous tubules were allowed to settle under unit gravity for 5 min, and the supernatant containing the interstitial cells were removed. The tubules were resuspended again in the medium and allowed to resettle and supernatants were collected. Supernatants were pooled together and transferred the Leydig cells enriched media into falcon tubes. The tube was inverted for 10 min and then filtered using nylon mesh. RBCs were removed by washing with 0.84% NH4Cl for 15 min. Macrophages present in the pellet were removed by incubating the pellet in Falcon Petri dish containing 8 ml of complete media (Dulbecco's Modified Eagle's Medium, Hams F12 nutrient mixture in 1:1 ratio and 10% fetal bovine serum [FBS]) for 45 min. Leydig cells were collected using the micropipette without disturbing the macrophages that settled at the bottom of Petri dish. Finally, Leydig cells were collected and counted using Neubauer chamber, cryopreserved using 95% of FBS and 5% of DMSO. The cells were counted using a hemocytometer and the purity was assessed by histochemical staining for the β3-hydroxy steroid dehydrogenase (β3-HSD) using the procedure described by Sharpe and Cooper.\textsuperscript{[43]} It was observed that more than 95% of the cells were positively stained for Leydig cells [Figure 1].
Determination of steroidogenic enzymes, enzymatic and nonenzymatic antioxidants in testis and isolated Leydig cells

3β-HSD,[44] 17β-HSD,[44] glucose-6-phosphatedehydrogenase(G-6PD),[43] lactate dehydrogenase,[46] superoxide dismutase (SOD),[47] catalase (CAT),[48] lipid peroxidation (LPO),[49] glutathione-S-transferase,[50] reduced glutathione (GSH),[51] hydroxyl radical,[52] and γ-glutamyl transpeptidase[53] were assayed according to the standard procedures.

Histopathological studies

At the end of the experimental schedule, one testis from each rat was taken and fixed in Bouin’s fluid, and processed, and the paraffin sections of 4 mm were stained with hematoxylin and eosin stain and then examined under microscope.

Statistical analysis

The data were computed using Prism GraphPad software (GraphPad Company, USA) program version 6.0 and presented as mean ± standard deviation if applicable. Statistical analysis was performed using one-way analysis of variance method, followed by Student’s t-test. The significance of differences was set at P < 0.05.

RESULTS AND DISCUSSION

Recent studies have reported the toxicity of piperine in testis and epididymis of male rats after treating them with different doses.[28-30] However, there is no information available about the effect of piperine for one complete spermatogenic cycle, i.e., 48 days. In addition, the effect of piperine after the withdrawal period, i.e., reversibility of piperine’s activity, is not reported. Here, we report the effect of piperine on male albino rats after a treatment period of 60 days and drug-free withdrawal period of 60 days. Various parameters related to spermatogenesis and male fertility were investigated and is discussed below.

Effect of piperine on body and organ weights of rats

Rats treated with piperine at a dose of 10 mg/kg (group ED, E4D) for 60 days have significantly reduced testicular weights. It is already reported that the weight of the reproductive organs always give a good measure of the degree of spermatogenesis in rats[46] moreover, it is suggested that significant decrease or increase in the absolute or relative weight of an organ after administration of a drug indicates the toxicity of the particular drug.[56,57] Hence, weights of the testis and prostate were also measured. The observed decrease in testicular weights in groups treated with piperine (ED, E4D) returned to normal levels after the drug-free withdrawal period. The decrease in the testicular weight after the daily treatment of piperine indicates the toxic effect of piperine on rat testis. No significant differences in the testis index were observed in all the treatment groups (ED, E4D, and E7D) compared to the control group [Table 1]. However, piperine caused a significant increase in the GSI in the group ED treated with piperine at a dose of 10 mg/kg b.w. for 60 days. GSI is inversely proportional to the reproductive efficiency of the rats[59] and testicular coefficient (TCT) value, which is directly proportional to reproductive toxicity in testes.[58] A marginal increase in the GSI in group E4D and E7D was observed after the treatment with piperine for 60 days. GSI and TCT in all the treated groups returned to normal levels after the drug-free withdrawal period of 60 days [Table 1]. Decrease in the prostatic weight in the piperine-treated groups can be correlated with the deregulation of the steroid hormone status within the prostate gland[59] in comparison with the control group. Weights of the prostates were back to normal in all the groups after the withdrawal period; it may be due to the return of normal function in all the accessory organs [Table 1].

Effect of piperine on serum and testicular hormonal status of rats

No significant differences in the serum testosterone (T) levels were observed in all the groups (ED, E4D, and E7D) treated with piperine at a dose of 10 mg/kg, compared to the control group even after 60 days. However, significant reduction in the intra-testicular testosterone was observed in rats treated with piperine in group ED and E4D. This could be due to the nonspecific inhibition of NADPH-dependent cytochrome P450 enzyme, which plays an important role in the steroid hormone synthesis pathway through catalyzing the cholesterol side chain cleavage and other important hydroxylation reactions.[60-62] This decrease in

Table 1: Effect of piperine on rat body and organ weights

| S.No | Parameter                  | Phase 1                        | Phase 2                        |
|------|----------------------------|--------------------------------|--------------------------------|
|      | Control                    | ED                             | E4D                            | E7D |
| 1    | Body weight Phase (g)      | 307±13.8                       | 275±6.89                       | 286±8.72 | 291±11.1 | 418±13.1 | 390±23.9 | 393±18.1 | 381±18.5 |
| 2    | Testicular weight (g)      | 3.16±0.14                      | 2.46±0.09*                     | 2.55±0.38* | 2.87±0.10 | 3.02±0.08 | 2.63±0.15 | 2.71±0.16 | 3.02±0.13 |
| 3    | Testis Index               | 0.52±0.05                      | 0.66±0.01                      | 0.52±0.02 | 0.53±0.02 | 0.64±0.03 | 0.62±0.02 | 0.59±0.03 | 0.63±0.02 |
| 4    | Gonadosomatic index        | 0.26±0.02                      | 0.30±0.01*                     | 0.26±0.03 | 0.26±0.01 | 0.36±0.01 | 0.37±0.02 | 0.43±0.03 | 0.44±0.02 |
| 5    | Testicular Co-efficient    | 1.02±0.05                      | 1.2±0.04*                      | 1.09±0.06 | 1.03±0.04 | 0.72±0.03 | 0.74±0.05 | 0.87±0.07 | 0.89±0.04 |
| 6    | Prostate weight (g)        | 0.57±0.06                      | 0.26±0.04*                     | 0.35±0.04 | 0.39±0.06 | 0.66±0.03 | 0.51±0.02 | 0.50±0.06 | 0.61±0.02 |

Values expressed as mean±SD, n=6; Control: Group treated with vehicle alone; ED: Treated with piperine 10 mg/kg body wt daily; E4D: Treated with piperine 10 mg/kg body wt on every 4th day; E7D: Treated with piperine 10 mg/kg body wt on every 7th day. Phase 1-Treatment period, Phase II-Withdrawal period; *P<0.05.
Effect of piperine on functional status of reproductive organs of rats

In our previous study, we have demonstrated the reversible spermatotoxic effect of piperine on male albino rats. Piperine significantly impaired the testicular function of rats in the group ED and E4D through elevating the testicular cholesterol levels, resulting in the inhibition of the steroidogenesis after the treatment period. A slight increase in the testicular cholesterol level was observed in the group E7D. Elevated cholesterol levels were returned to normal levels after the drug-free withdrawal period. Inhibition of the steroidogenesis was also evidenced by a decrease in the activity of two crucial enzymes, i.e. 3β-HSD and 17β-HSD, which are responsible for testosterone synthesis in group ED and E4D.[69] [Table 3].

Administration of piperine caused a significant disruption in the membrane lipid bilayers of the plasma membrane, resulting in the leakage of ALP into the extracellular matrix, which results in the functional impairment of the testicular cells. A marked decrease in ALP activity in groups ED and E4D after the treatment period was observed as compared to the control group. This effect of the piperine may also be attributed due to its fungicidal properties.[68,69] Along with the decreased level of ALP, a decrease in the total ACPs was also observed in groups treated with piperine (ED and E4D), which confirms the piperine-mediated inhibition of steroidogenesis in rats. The degree of steroidogenesis is always dependent on the concentration of ACP.[70] Hence, a decrease in the level of ACP by piperine decreases the steroidogenesis. Piperine negatively affected the development of spermatocytes by decreasing the detachable ACP of lysosomal origin.[71] However, after the drug-free withdrawal period, both the ALP and ACP levels were restored to normal in all the piperine-treated groups.

A decrease in testicular γ-glutamyl tranpeptidase (γ-GT) activity was observed in piperine treated groups indicating the dysfunction of the Sertoli cell. This could be due to the inhibition of androgen receptor by piperine as the function of γ-GT depends upon the regulation of androgen receptor. In vitro study also supports the same.[4,72,73]

Lactate dehydrogenase (LDH) plays a crucial role in the production of energy during spermatogenesis, and it is also necessary for the proper functioning of Sertoli cells.[74,75] LDH activity was found to be inhibited in groups ED and E4D, indicating suppression of testicular function. Further, piperine caused dysfunction of Leydig cells by inhibiting the activity of G-6-PD in groups ED and E4D after the treatment period. Determination of G-6-PD activity gives a good measure of the functional status of Leydig cells.[76] The activity of γ-GT, LDH, and G-6-PD was restored to normal level after the drug-free withdrawal period [Table 4].

Effect of piperine on enzymatic and nonenzymatic antioxidant status of rat testis

Oxidative damage caused to the rat testis can be shown by a decrease in the activities of two major antioxidant enzymes, namely, SOD and GSH. Oxidative damage caused to the rat testis can be shown by a decrease in the activities of two major antioxidant enzymes, namely, SOD and GSH.
CAT, to a significant extent in the groups (ED, E4D and E7D) after the piperine treatment period of 60 days. SOD converts the superoxide radicals to hydrogen peroxide ($H_2O_2$) and CAT is crucial for the conversion of $H_2O_2$ into water. These two enzyme systems act together to prevent oxidative damage. Piperine is known to activate the membrane LPO of lipids in rat tests. In this study, significant increases in the malondialdehyde (MDA) levels were observed in the groups ED and E4D treated with piperine for 60 days. Increase in the MDA content of the tissue results in excessive generation of free radicals. Piperine-mediated oxidative damage through the excessive generation of reactive oxygen species (ROS) was supported with an increase in the hydroxyl radical content in the testes of the rats treated with piperine for 60 days (groups ED and E4D). Testicular toxicity of piperine can be correlated with a significant depletion in the reduced GSH levels in groups ED and E4D after the treatment period. Perturbation of intracellular GSH levels leads to a negative alteration in the cellular metabolism. This results in decreased detoxification potential and redox imbalance in the testicular tissue. Moreover, declined levels of GSH may be associated with an enhanced LPO. Table 4.

The extent of oxidative damage caused to the testis can be shown by a decrease in the activity of glutathione-S-transferase (GST) in the groups ED and E4D treated with piperine for 60 days. GST plays a vital role in the adaptive defensive mechanisms and works against the free radical induced oxidative damage and eliminates the toxic products by catalyzing the conjugation reactions. On the contrary to its catalytic ability, GST is important for the functioning of the sperm. All the above-mentioned altered activities of enzymes were restored to normal levels after the drug-free withdrawal period of 60 days [Table 4].

**Table 4: Effect of piperine on biochemical status of rat testis**

| S.No | Parameter          | Phase 1                              | Phase II                              |
|------|--------------------|--------------------------------------|---------------------------------------|
|      |                    | Control | ED | E4D | E7D | Control | ED | E4D | E7D |
| 1    | Super oxide dismutase | 1.18±0.03 | 0.67±0.03 * | 0.85±0.04 | 0.91±0.04 | 0.88±0.05 | 0.76±0.04 | 0.74±0.03 | 0.80±0.04 |
| 2    | Catalase            | 0.46±0.01 | 0.19±0.03 | 0.29±0.03 | 0.41±0.05 | 0.14±0.02 | 0.13±0.05 | 0.16±0.02 | 0.11±0.02 |
| 3    | Lipid peroxidation  | 1.21±0.06 | 2.85±0.02 * | 0.21±0.04 | 1.62±0.01 | 1.09±0.36 | 0.93±0.17 | 1.06±0.22 | 1.14±0.13 |
| 4    | Glutathione -S-transferase | 1.10±0.24 | 0.33±0.16* | 0.83±0.13 | 0.60±0.17 | 0.59±0.14 | 0.60±0.02 | 0.51±0.10 | 0.69±0.09 |
| 5    | Reduced Glutathione | 5.47±0.53 | 3.07±0.49* | 4.52±0.24 | 5.1±0.44 | 3.98±0.31 | 4.4±0.47 | 3.98±0.06 | 4.8±0.04 |
| 6    | Hydroxyl Radical   | 1.14±0.37 | 2.98±0.05* | 1.13±0.17 | 1.27±0.24 | 1.14±0.04 | 1.16±0.05 | 1.19±0.07 | 1.13±0.04 |
| 7    | γ-Glutamyl transpeptidase | 1.53±0.08 | 0.74±0.08* | 0.80±0.17 | 1.56±0.15 | 1.37±0.13 | 1.41±0.22 | 1.53±0.16 | 1.36±0.21 |
| 8    | Lactate Dehydrogenase | 0.71±0.10 | 0.38±0.03* | 0.56±0.04 | 0.64±0.03 | 0.54±0.05 | 0.48±0.36 | 0.61±0.03 | 0.58±0.03 |
| 9    | Glucose-6-Phosphate dehydrogenase | 0.54±0.01 | 0.27±0.08* | 0.39±0.01 | 0.34±0.02 | 0.37±0.03 | 0.39±0.03 | 0.36±0.01 | 0.37±0.02 |

Units: SOD: units/mg protein; CAT: H$_2$O$_2$ Consumed/min/mg protein; LPO: nmoles of MDA formed/mg protein; GST: nmoles of CDNB-GSH complex formed/min/mg protein; GTP: μmoles of p-nitroaniline formed/min/mg protein; G-6-P: units/mg protein, GSH-μg/mg protein; LDH- mU/ml, Hydroxyradical - μmoles/min/mg protein.

**Effect of piperine on antioxidant status of the Leydig cells**

Piperine significantly disrupted the antioxidant defensive mechanism of Leydig cells in groups ED and E4D by decreasing the activity of SOD and CAT after the 60 days treatment. SOD protects the Leydig cells from the free radical induced damage by converting superoxide anions into hydrogen peroxide ($H_2O_2$) and impairs the LPO. Whereas, CAT eliminates the lipid, protein, and DNA destructive $H_2O_2$ by its catalytic activity. Increase in the ROS production can be shown with concomitant increase in MDA levels in groups ED and E4D. The decreased activity of SOD and CAT and increased MDA content were reinstated to normal levels after the drug-free withdrawal period of 60 days [Table 5].

**Histopathological studies**

Histology section of the group ED shows many desquamated spermatozoa with a decrease in the thickness of germ layer. The tubules showed both Type 1 and Type 2 spermatogonia and the rest composed of mostly mature spermatozoa. Primary spermatocytes are reduced in number compared to the control group. Section of the group E4D shows a decrease of 30% spermatozoa in the tubules, resulting in hypospermatogenesis. In contrary to this, section of group E7D shows a decrease of 10% spermatozoa in tubules, which represents mild hypospermatogenesis compared to the control group [Figure 2]. All the pathological changes appeared after treatment period returned to normal status after the drug-free withdrawal period of 60 days [Figure 3].

**CONCLUSION**

Compared to the synthetic molecules, natural and herbal products have been more successful in delivering safer drugs for the human kind.
The present study was aimed to evaluate the effect and reversibility of piperine mediated testicular toxicity in male albino rats. Various parameters related to male fertility such as weights of the testis and prostate, serum, and intra-testicular testosterone levels, and serum gonadotropin levels were measured. Furthermore, functional status of testis was ascertained by measuring the steroidogenesis-related parameters such as activity levels of enzymes 3β-hydroxy and 17β-HSD, ALP, ACPs, γ-GT, lactate dehydrogenase, and G-6PD. The extent of oxidative damage to testis caused by piperine treatment was monitored by measuring the activity levels of SOD, CAT, and GST enzymes; further, levels of MDA, ROS, and reduced glutathione levels were measured.

Our study result suggests that piperine disrupted the functional integrity of the testis by altering the germ cell markers, antioxidant status, and testicular hormones. These biochemical study results were further reinforced by histopathological observation of hypospermatogenesis on piperine treatment. However, the toxic effects of piperine were reversed after the drug-free withdrawal period of 60 days, suggesting reversible nature of piperine effect on male fertility. From the above results, it can be concluded that piperine can be used as a lead molecule for the development of reversible oral male contraceptive agent.

Financial support and sponsorship
Authors thank the Department of Biotechnology, Govt. of India, for the financial support for the DBT-IPLS program (Grant No : BT/PR14554/INF/22/125/2010), Pondicherry University, Puducherry, India. Authors also thank Professor T.G.Shrivatsav, NIHFW, Munirka, New Delhi for Providing the Testosterone and Progesterone Elisa kits.

Conflicts of interest
There are no conflicts of interest.

REFERENCES
1. Mruk DD. New perspectives in non-hormonal male contraception. Trends Endocrinol Metab 2008;19:57-64.
2. Pasqualotto FF, Lucon AM, Pasqualotto EB, Arap S. Trends in male contraception. Rev Hosp Clin Fac Med Sao Paulo 2003;58:275-83.
3. Vijayakumar RS, Nalini N. Piperine, an active principle from Piper nigrum, modulates hormonal and apo lipoprotein profiles in hyperlipidemic rats. J Basic Clin Physiol Pharmacol 2006;17:71-86.
4. Chinta G, Ramya Chandar Charles M, Klopacic I, Sollner Dolenc M, Periyasamy L, Selvaraj Coumar M. In silico and in vitro investigation of the Piperine’s male contraceptive effect: Docking and molecular dynamics simulation studies in androgen-binding protein and androgen receptor. Planta Med 2015;81:804-12.
5. Johri RK, Thusu N, Khajuria A, Zutshi U. Piperine-mediated changes in the permeability of rat intestinal epithelial cells. The status of gamma-glutamyl transpeptidase activity, uptake of amino acids and lipid peroxidation. Biochem Pharmacol 1992;43:1401-7.
6. Kulkami SK, Bhutani MK, Bishnoi M. Antidepressant activity of curcumin: Involvement of serotonin and dopamine system. Psychopharmacology (Berl) 2008;201:435-42.
7. Li S, Wang C, Li W, Koike K, Nikaido T, Wang MW. Antidepressant-like effects of piperine and its derivative, antiepilepsirine. J Asian Nat Prod Res 2007;9:421-30.
8. Li S, Wang C, Wang M, Li W, Matsumoto K, Tang Y. Antidepressant-like effects of piperine in chronic mild stress treated mice and its possible mechanisms. Life Sci 2007;80:1373-81.
9. Atal S, Agrawal RP, Vyas S, Phadnis P, Rai N. Evaluation of the effect of piperine per se on blood glucose level in alloxan-induced diabetic mice. Acta Pol Pharm 2012;69:965-9.
10. Atal S, Atal S, Vyas S, Phadnis P. Bio-enhancing effect of piperine with metformin on lowering blood glucose level in alloxan induced diabetic mice. Pharmacognosy Res 2016;8:56-60.
11. Mujumdar AM, Dhuley JN, Deshmukh VK, Ramam PH, Nalk SR. Anti-inflammatory activity of piperine. Jpn J Med Sci Biol 1990;43:95-100.
12. Park BS, Son DJ, Park YH, Kim TW, Lee SE. Antipla telet effects of acidamides isolated from the fruits of Piper longum L. Phytochemistry 2007;74:853-6.
13. Panda S, Kar A. Piperine lowers the serum concentrations of thyroid hormones, glucose and hepatic S’D activity in adult male mice. Horm Metab Res 2003;35:523-6.
14. Veerareddy PR, Vobalaboina V, Nahid A. Formulation and evaluation of oil-in-water emulsions...
of piperine in visceral leshmaniasis. Pharmazie 2004;59:194-7.
15. Kim SH, Lee YC. Piperine inhibits eosinophil infiltration and airway hyperresponsiveness by suppressing T cell activity and Th2 cytokine production in the ovalbumin-induced asthma model. J Pharm Pharmacol 2009;61:153-9.
16. Bajad S, Bedi KL, Singla AK, Johri RK. Antidornhoozel activity of piperine in mice. Planta Med 2001;67:284-7.
17. Samvukty A, Shetty AV, Dakshinamurthty G, Barik MM, Johnson GL, Webb B, et al. Piperine, a bioactive component of pepper spice exerts therapeutic effects on androgen dependent and androgen independent prostate cancer cells. PLoS One 2013;8:e65889.
18. El Hamss R, Idacomar M, Alonso-Moraga A, Muñoz Serrano A. Antimutagenic properties of bell and black peppers. Food Chem Toxicol 1993;31:41-7.
19. Koul IB, Kapil A. Evaluation of the liver protective potential of piperine, an active principle of black and long peppers. Planta Med 1993;59:413-7.
20. Zhao JQ, Du GZ, Xiong YC, Wen YH, Bhadurania M, Niranla SK. Attenuation of benzyll induced hepatorenal dysfunction and oxidative stress in rodents by combined effect of gallic acid and piperine. Arch Pharm Res 2007;30:1576-83.
21. Badmavd V, Majid M, Nirkus PE. Piperine, an alkaloid derived from black pepper increases serum response of beta carotene during 14 day oral beta carotene supplementation. Nutr Res 1999;19:381-8.
22. Niranla SK, Bhadurania M, Mathur R, Mathur A. Influence of alpha-tocopherol, propolis and piperine on therapeutic potential of tilmor against benzyll induced toxic manifestations. J Appl Toxicol 2008;28:44-54.
23. Lambert JD, Hong J, Kim DH, Mishin VM, Yang CS. Piperine enhances the bioavailability of the tea polyphenol 1-L-epigallocatechin-3-gallate in mice. J Nutr 2004;134:1848-62.
24. Chinta G, Saffulla BS, Mohane SC, Latha P. Piperine: A comprehensive review of its preclinical and clinical investigations. Curr Bioact Compd 2015;11:156-69.
25. Han HK. The effects of black pepper on the intestinal absorption and hepatic metabolism of drugs. Expert Opin Drug Metab Toxicol 2011;7:721-9.
26. Padi UK, Singh A, Chakraborty AK. Role of piperine as a bioavailability enhancer. Int J Recent Adv Pharm Res 2011;4:363-8.
27. Gupta RS, Kachhava JB, Chaudhary R. Antispasmotogenic, antidiarrhoeal activities of Alloizia lebeck (L.) Bent bark extract in male albino rats. Phytomedicine 2008;15:277-83.
28. Malini T, Manimaran RR, Arunakaran J, Anudhas MM, Govindaraju P. Effects of piperine on tests of albino rats. J Ethnopharmacol 1999;64:219-25.
29. D’croz SC, Mathur PP. Effect of piperine on the epididymis of adult male rats. Asian J Androl 2005;7:363-8.
30. D’croz SC, Vaiithinathan S, Saradha B, Mathur PP. Piperine activates testicular apoptosis in adult rats. Indian J Endocrinol Metab 2011;15:e184-90.
31. Castillo F, Hernández D, Gallegos R, Rodríguez, Aguilar CN. Antifungal properties of bioactive triterpenoids from Thaspia montana (L.) Benth bark extract in male albino rats. Phytomedicine 2006;13:277-83.
32. Chitra KC, Latchoumycandane C, Mathur PP. Chronic effect of endosulfan on the testicular stimulation of rat testis. Andrologia 1982;14:156-69.
33. Devasagayam TP, Tanachard U. Decreased lipid peroxidation in the testis following administration of a herbal tea mixture. J Pharmacol Toxicol 2008;3:464-70.
34. Simmons JE, Yang RS, Berman E. Evaluation of the nephrotoxicity of complex mixtures containing organics and metals: Advantages and disadvantages of the use of real-world complex mixtures. Environ Health Perspect 1996;103 Suppl 1:67-71.
35. Silva AA, Essica SD, de Oliveira RR, Silva RA, Valdemiro AS, Elizabeth NM. Evaluation of quantitative parameters of Leydig cell in diabetic adults. Acta Sci Biol Sci 2014;36:483-9.
36. Yuan L, Fan M, Zhan P. Dose-dependent effects of pentabrominated diphenyl ethers on sexual hormone and histology of male reproductive system in rats. J Biomed Sci Eng 2012;5:302-6.
37. Weber KS, Setchell KD, Stocco DM, Lepart EH. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate βα3-reductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. J Endocrinol 2001;170:591-9.
38. Miller WL. Molecular biology of steroid hormone synthesis. Endocr Rev 1988;9:295-318.
39. Reen RK, Singh J. In vitro and in vivo inhibition of pulmonary cytochrome P450 activities by piperine, a major ingredient of piper species. Indian J Exp Biol 1991;29:568-73.
40. Reen RK, Wiebel FJ, Singh J. Piperine inhibits aflatoxin B1-induced cytotoxicity and genotoxicity in V79 Chinese hamster cells genetically engineered to express rat cytochrome P450B1. J Ethnopharmacol 1997;58:165-73.
41. Alveanga TA, Andersen ML, Tufik S. Influence of progesterone on sexual performance in male rats. J Sex Med 2010;7:2435-44.
42. Hanis ID, Franzcek C, Roth L, Meacham RB. Fertility and the aging male. Rev Urol 2011;13:e184-90.
43. Chinta G, Periyasamy L. Reversible anti-spermatozoenct effect of piperine on epididymis and seminal vesicles of albino rats. Drug Res (Stuttg) 2016;66:420-6.
44. Kaur R, Dhanju CK, Kaur K. Effects of dietary selenium on biochemical composition of rat testis. Indian J Exp Biol 1999;37:509-11.
45. Ghosh S, Dasgupta S. Gentamicin induced inhibition of steroidogenic enzymes in rat testis. Indian J Physiol Pharmacol 1999;43:247-50.
46. Hanas KW, Kumanan B. Effect of marcozeb on the specific activities of testicular phosphates and protective role of Vitamin c in albino rats. BEPLS 2013;2:66-61.
47. Castillo F, Hernández D, Gallegos R, Rodriguez, Aguilar CN. Antifungal properties of bioactive compounds from plants, fungicides for plant and animal disease. In: Dhanasekaran D, editor. Fungicides for Plant and Animal Diseases. China: In Tech; 2012. p. 1-27.
48. Mathur PP, Chappadhyay S. Involvement of lysosomal enzymes in flutamide-induced stimulation of rat testis. Andrologia 1982;14:171-6.
49. Chitra KC, Latchoumycandane C, Mathur PP. Chronic effect of endosulfan on the testicular
functions of rat. Asian J Androl 1999;1:203-6.

72. Olayinka E, Ore A. Hepatotoxicity, nephrotoxicity and oxidative stress in rat testis following exposure to haloxyfop-p-methyl ester, an aryloxyphenoxypropionate herbicide. Toxics 2015;3:373-89.

73. Sherins RJ, Hodgen GD. Testicular gamma glutamyl-transpeptidase: An index of Sertoli cell function in man. J Reprod Fertil 1976;48:191-3.

74. El-Kashoury AA. Influence of subchronic exposure of profenofos on biochemical markers and microelements in testicular tissue of rats. Nat Sci 2009;7:16-29.

75. Zhang B, Lin S. Effects of 3,4-dichloroaniline on testicle enzymes as biological markers in rats. Biomed Environ Sci 2009;22:40-3.

76. Heywood L, Blackshaw A. Lactate-dehydrogenase activity in the rat testis: A comparison between fluorometric assay of freeze-dried sections and histochemical localization with phenazine methosulphate. J Histochem Cytochem 1978;26:967-72.

77. Kalender S, Uzun FG, Demir F, Uzunhisarcikli M, Aslanturk A. Mercuric chloride-induced testicular toxicity in rats and the protective role of sodium selenite and vitamin E. Food Chem Toxicol 2013;55:456-62.

78. Rahal A, Kumar A, Singh V, Yadav B, Triwari R, Chakraborty S, et al. Oxidative stress, prooxidants, and antioxidants: The interplay. Biomed Res Int 2014;2014:761264.

79. Vigueras-Villaseñor RM, Ojeda I, Gutiérrez-Pérez Q, Chavez-Saldaña M, Cuevas Q, Maria DS, et al. Protective effect of a-tocopherol on damage to rat testes by experimental cryptorchidism. Int J Exp Pathol 2011;92:131-9.

80. Maneesh M, Jayalekshmi H, Dutta S, Chakrabarti A, Vasudevan DM. Role of oxidative stress in ethanol induced germ cell apoptosis – An experimental study in rats. Indian J Clin Biochem 2005;20:62-7.

81. Khan RA. Protective effects of Launaea procumbens on rat testis damage by CCl4. Lipids Health Dis 2012;11:103.

82. Gopalakrishnan B, Aravinda S, Pawshe CH, Totey SM, Nagpal S, Salunke DM, et al. Studies on glutathione S-transferases important for sperm function: Evidence of catalytic activity-independent functions. Biochem J 1998;329(Pt 2):231-41.

83. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev 2008;1:15-24.

84. Gautam DK, Misro MM, Chaki SP, Sehgal N. H2O2 at physiological concentrations modulates Leydig cell function inducing oxidative stress and apoptosis. Apoptosis 2006;11:39-46.