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Research Article

EFFECT OF PIPERINE ON GOAT EPIDIDYMAL SPERMATOZOA: AN *IN VITRO* STUDY

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ABSTRACT

Objective: Fertility control is a global issue in the perspective of health and economy worldwide. Many of the investigations were carried out to prove the anti-fertility effect of the traditional natural products in males. Until today, there is no such reversible contraceptive is available for males. Piperine is a versatile bioactive compound being used from the centuries to treat domestic animal illness as well as in humans. This study was aimed to evaluate the effect of piperine on goat epididymal spermatozoa in vitro.

Methods: Goat sperms were incubated with piperine at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L for 3 hrs. After 3 hrs parameters such as motility, viability, superoxide dismutase (SOD), Catalase (CAT) activities, and lipid peroxidation (LPO) level was monitored. Hypo-smotic swelling test and acridine orange test were performed.

Results: Significant decrease in motility, viability, SOD, CAT activities of goat epididymal sperm was observed after 3 hrs of incubation with piperine. Significant increase in LPO levels was found after 3 hrs of incubation with piperine at above mentioned doses. Piperine produced significant disruption in the functional integrity and also damaged the DNA of goat epididymal sperm.

Conclusion: From the above results, we can conclude that increase in the oxidative damage and functional disruption of sperm by piperine might be one of the reasons for its anti-fertility activity.

Keywords: Piperine, Anti-fertility agent, Superoxide dismutase, Catalase, Lipid peroxidation.

INTRODUCTION

Increasing human population continues to be a significant contributor to environmental degradation and human suffering worldwide. Hence, investigators are more focused on the development of reversible male contraceptives without any adverse effects. Until today, no such ideal contraceptive is available for use. However, the increase in importance of phytochemical in male contraceptive practices has been reported in different animal models [1-3]. Testes perform two typical functions for the perpetuation of life events, called steroidogenesis and spermatogenesis. Spermatogenesis is a highly versatile and complex process, takes place within the seminiferous tubules of the testis with the help of Sertoli cells, leading to the formation of mature spermatozoa from undifferentiated stem cells [4] where Leydig cells are the site of steroidogenesis [5]. Luminal compartment of the epididymis stores the spermatozoa until ejaculation and specifically prepares the sperm for fertilization by providing the essentials in terms of temperature, oxygen tension, pH and an available energy substrate [6]. The epididymal duct produces the morphological, biochemical, physiological, and functional changes to the structures of the spermatozoa through a process known as epididymal maturation, which converts the spermatozoa into fertilization- competent cells [7-9]. Oxidative stress (OS) has been identified as one factor that affects fertility status and thus has been extensively studied in recent years. OS is a consequence of an imbalance between the production of reactive oxygen species (ROS), which are present as free radicals and body's antioxidant defense mechanisms [10]. The mechanism by which ROS disrupt sperm function is believed to involve the peroxidation of unsaturated fatty acid in sperm plasma membrane. Sperm is particularly susceptible to oxidative damage due to its unique structural composition of high polyunsaturated fatty acid content in its plasma membrane [11-13]. Attack by ROS, particularly H_2O_2 initiates a peroxidative chain reaction during which the unsaturated fatty acids are converted to lipid peroxides (LPO), which in the presence of transition metal such as iron and copper, can break down further to yield small carbonyl compounds such as malondialdehyde (MDA) [14]. Fortunately, the epididymis has been enriched with an antioxidant defense system that protects the sperm during their voyage through the caput to caudal region of epididymis and thereby facilitates their maturation process [15]. Measurement of lipid peroxidation (LPO) level has been used as a diagnostic tool for the analysis of the generation of ROS/second [16]. Hence, a greater understanding of the function and biochemical properties of the epididymis will allow for the development of male contraceptive agents [17] and may lead to the development of therapeutic agents to treat certain types of infertility.

In order to achieve the production of male contraceptive without any detrimental effects, researchers are more focused on plants and plant based products that have been attributed to the anti-spermatogenic and anti-steroidogenic properties. One among them is piperine (1-peperoylpiperidine), the primary pungent alkaloid (Fig. 1) [18] in black peppercorns derived from the fruit bodies of black pepper (Piper nigrum), long pepper (Piper longum) and piper species (Family: Piperaceae), is commonly ingested in many diets throughout the world.

However, recent literatures indicate that seeds of P. nigrum caused the alterations in the histo architecture of testis and epididymis of mice [19]. Goat sperm has been taken into consider in most of the in vitro studies, especially assisted reproductive technology, represent a useful tool to select the most suitable donors for semen cryopreservation [20]. Piperine is an alkaloid, a member of the family Piperaceae. The genus piper has more than 1000 species but the most well-known species are P. nigrum, P. longum. Piperine is commonly used as a spice all over the world which possesses pharmacological properties, such as antipyretic, analgesic and anti-inflammatory activities and has been used in ayurvedic medicine for the treatment of various disease for thousands of years [21]. For many years, it has

been known that piperine can interfere with the reproductive process. In addition to its common utility as a gustatory enhancer, it has found many rather diverse applications ranging from brandy flavoring to its use as an insecticide [22]. Piperine is reported to exhibit anti-diarrheal and gastro protective properties in rodents [23,24]. On being so, piperine is reported to induce sterility in laboratory male mice [25]. It has been shown to cause damage to the germ cells and seminiferous tubules, along with the disruption of spermatogenesis when administered orally for 30 days and also elevated the level of serum gonadotropin by impairing the feedback signal, which results in decreasing intratesticular testosterone concentrations [26]. Studies on male rats have shown that piperine decrease the activity of the antioxidant enzymes and sialic acid levels, which result in the impairment of sperm motility, viability and count [27]. It also suppresses the level of antioxidant enzymes and increases LPO in testis and epididymis. Piperine also activates caspase 3 and fas apoptotic proteins in testicular germ cells when piperine administered to male Wistar rats at doses of 10 mg and 100 mg for 30 days [28]. An in vitro study on hamster sperm showed that the piperine interferes with acrosome reaction through the inhibition of calcium influx by stimulation of efflux, thereby impairing fertility [29]. Studies on Swiss albino female mice reported that intrauterine injection of piperine caused the total absence of implants in either of uterine horns (16.66%) or one of the horns (33%) [30]. The effect of piperine on goat epididymal sperm has not been done yet in vitro. Hence, the present study was aimed to explore the effect of piperine on sperm motility, viability and anti-oxidant system of goat epididymal sperm.

METHODS

Chemicals

Piperine was purchased from Sigma Aldrich, USA. All other chemicals used were of analytical grade and were obtained from local commercial vendors.

Collection of goat sperm

Goat testis was collected from local slaughter house immediately after killing and transported in wide mouth bottle containing ringer's phosphate solution (RPS), which composed of 119 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate, penicillin 50 units/mL (pH: 6.9). Briefly, epididymis was cut into 4-5 pieces with a sharp razor blade and dispersed in a modified RPS with gentle stirring. The sperms obtained were washed several times in RPS and centrifuged at 225 g for 10 minutes. An aliquot of the sperm suspension were homogenized in cold RPS medium using glass Teflon homogenizer. An aliquot was centrifuged at 800 g for 20 minutes at 4°C. The supernatant was used for biochemical assays [31]. The epididymal sperm were diluted with RPS medium and counted on Neubauer chamber hemocytometer using trypan blue as a staining dye. Sperms were incubated at the density of 0.3 $\times 10^6$ for 100 µL.

Experimental design

Group I: 100 µL of epididymal sperm samples were incubated with 100 µL of RPS at 32°C for 3 hrs as a control without any treatment.

Group II: 100 µL of epididymal sperm samples were incubated with 100 µL of 40 µmol/L piperine at 32°C for 3 hrs.

Group III: 100 µL of epididymal sperm samples were incubated with 100 µL of 60 µmol/L piperine at 32°C for 3 hrs.

Group IV: 100 µL of epididymal sperm samples were incubated with 100 µL of 80 µmol/L piperine at 32°C for 3 hrs.

Group V: 100 µL of epididymal sperm samples were incubated along with 100 µL of 100 µmol/L piperine at 32°C for 3 hrs.

After incubation, an aliquot of sperm suspension was placed in the Neubauer hemocytometer. The percentages of motile sperms were counted under light microscope. The viability of epididymal sperm was determined by eosin. The data were expressed in percentage of total sperm. All the sperm parameters were repeated thrice.

Evaluation of sperm parameters

Sperm parameters such as motility, viability had been evaluated by obtaining spermatozoa from the cauda epididymis according to the method described by Malini et al., 1999 [26].

Biochemical assays

After 3 hrs of incubation, the biochemical assays of superoxide dismutase (SOD) [32], catalase (CAT) [33] activities and LPO level [34] were performed.

Hypo-osmotic swelling (HOS) test and acridine orange test

HOS test [35] and acridine orange test [36] was performed according to the standard protocols mentioned in the literature.

Statistical analysis

The data were computed using prism graph pad software program. Version 6.0 and presented as mean ± standard deviation of six samples from each group. Statistical analysis was performed using the Student's t-test. Significance of differences was set at $p<0.05$.

RESULTS

Effect of piperine on sperm viability of goat epididymal sperm

Piperine exhibited a significant reduction in sperm viability of goat spermatozoa at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L (Table 1).

Effect of piperine on sperm motility of goat epididymal sperm

Piperine exhibited a significant reduction in sperm motility of goat spermatozoa at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L (Table 1).

Effect of piperine on the structural integrity of goat epididymal sperm

Structural integrity of goat sperm was significantly disturbed by piperine at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L (Table 2 & Fig. 2).

Effect of piperine on DNA of goat epididymal sperm

Significant damage of DNA was achieved by piperine at the doses of piperine at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L (Table 2 & Fig. 3).

Effect of piperine on the activity of the SOD and CAT

Piperine significantly decreased the activity of superoxide dismutase and CAT at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L (Table 2).

Table 1: Effect of piperine on sperm viability and motility of goat epididymal sperm

Parameters	Control	Concentration of piperine (µmol/L)					
		10	20	40	60	80	100
Viability (%)	72±2.0	67±2.6	61±2.8	56±1.4*	54±1.5*	51±2.5*	41±1.6*
Motility (%)	63±1.8	57±2.0	55±1.9	40±1.8*	37±1.6*	35±2.4*	30±1.5*

Mean±SEM per Group, *p<0.05, SEM: Standard error of the men

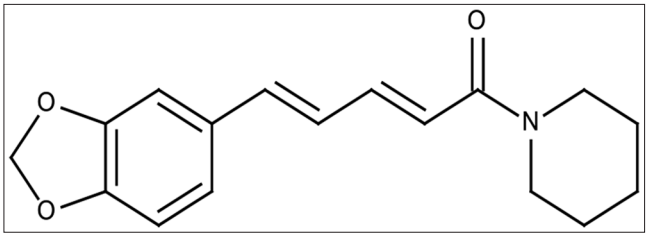

Fig. 1: 1-(5-[1, 3-benzodioxol-5-yl]-1-oxo-2, 4-pentadienyl) piperidine

Effect of piperine on LPO

Piperine showed significant increase in LPO levels at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L (Table 2).

DISCUSSION

OS is one of the most important factor [37] contributing to the poor semen quality. OS always associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as ROS. Excessive and uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to OS,

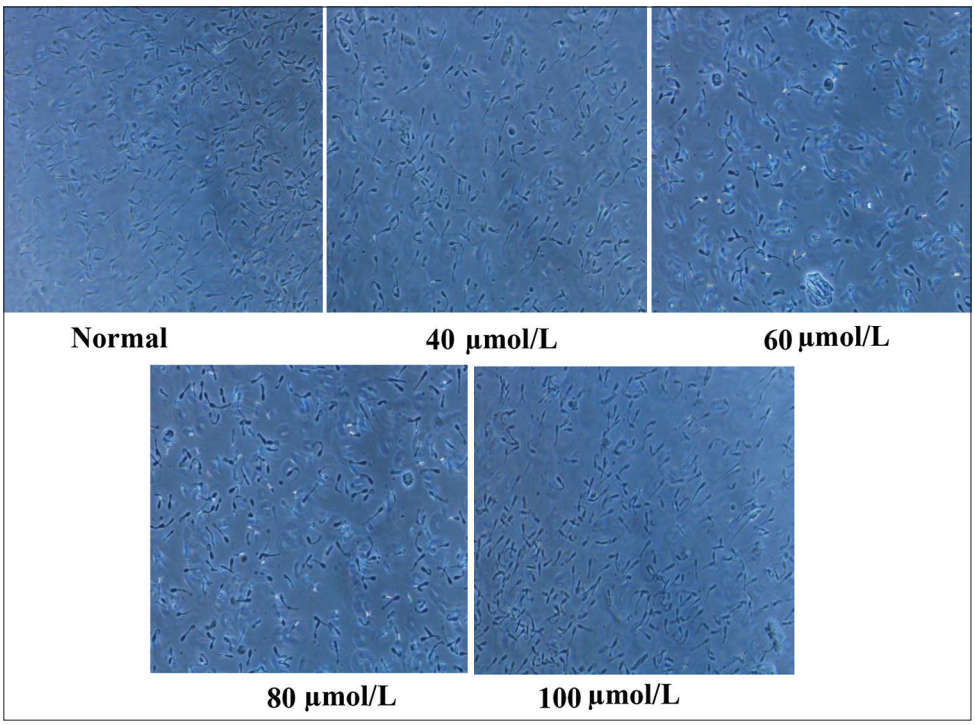

Fig. 2: Hypo-osmotic swelling test

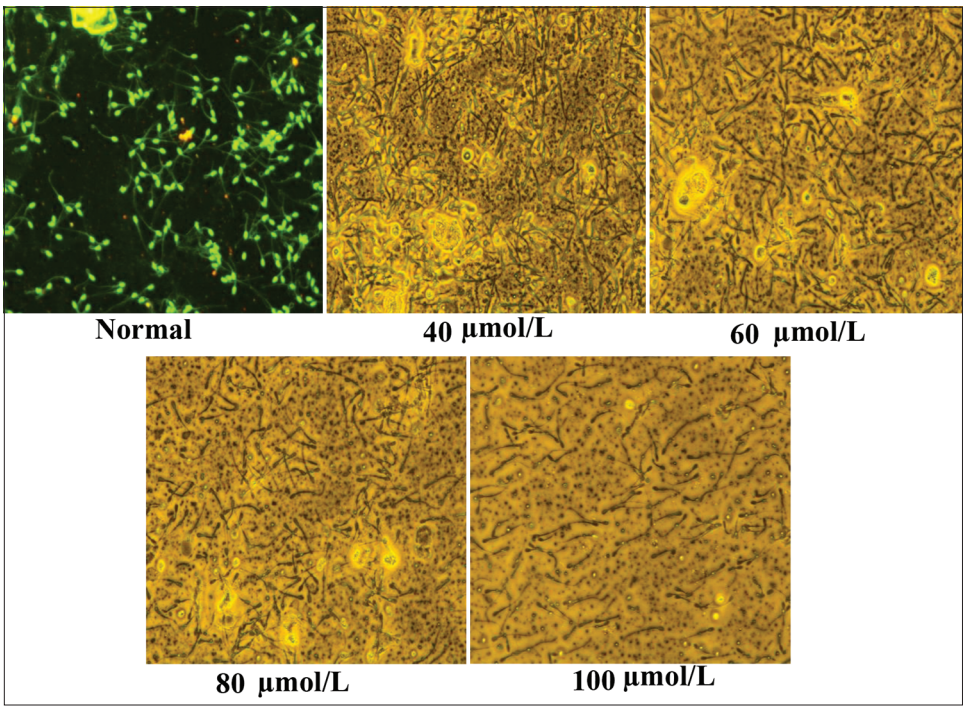

Fig. 3: Acridine orange test

Table 2: Effect of piperine on HOS, acridine orange and anti-oxidant system of goat epididymal spermatozoa

Parameters	Control	Concentration of piperine (µmol/L)			
		40	60	80	100
HOS	66±1.3	58±1.7*	54±1.8*	52±1.9*	47±1.2*
Acridine orange test	58±1.5	50±1.3*	47±1.9*	45±1.7*	40±1.4*
SOD	11±0.5	8.1±0.4*	7.8±0.2*	7.5±0.62*	6.8±0.7*
CAT	61±1.1	52±2.0*	47±1.9*	43±2.0*	40±2.1*
LPO	6.2±0.7	16±1.0*	17±1.5*	20±2.7*	25±2.2*

Mean±SEM per Group.*=p<0.05, HOS: Hypo-osmotic swelling, SOD: Superoxide dismutase, LPO: Lipid peroxidation, CAT: Catalsae, MDA: Malondialdehyde, SEM: Standard error of the men, Units: Acridine orange test: Percentage of green sperms, HOS Test: Percentage of functional sperms. CAT-mm of H_2O_2 consumed per minutes/mg protein, SOD-nmoles of pyrogallol oxidized per minutes/mg protein, LPO-nm of MDA formed per minutes/mg protein

which is harmful to spermatozoa. All cellular components, including lipids, proteins, nucleic acids, and sugars are potential targets of OS [37].

Significant reduction in the motility and viability was observed with the treatment of piperine at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L. This might be due to the generation of free radicals by the action of piperine since free radicals are known to reduce sperm motility and viability and thus may contribute to male infertility [38]. Test HOS is the most widely used tool for the analysis of functional integrity of sperm membrane [35]. Significant disruption in functional integrity of sperm membrane had been achieved by piperine at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L. Acridine orange test is well-known for the differentiation of fertile (green) and non-fertile (red) sperms using fluorescence visualization at 490 nm [36]. Piperine significantly reduced the percentage of fertile sperms in all the treated groups. SOD usually dismutases the superoxide anion radical into hydrogen peroxide, CAT removes the hydrogen peroxides formed during the metabolic reactions [32,33]. Among all the anti-oxidant enzymes CAT has one of the highest turnover; one molecule of CAT can convert millions of molecules of hydrogen peroxide to water and oxygen per second [39]. Treatment with piperine at the doses of 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L was observed with significant reduction of both enzyme activities. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids. Therefore sperm cells are particularly susceptible to the damage induced by excessive ROS release. LPO plays a major role in the etiology of defective sperm function. This may lead to the onset of male infertility via the mechanism involving the induction of peroxidative damage to plasma membrane of sperm [38]. LPO level is an indicator for the OS. MDA, a LPO product is generated in tissues by free radical injury, usually measured by thiobarbituric acid reactivity and has been considered as sensitive index of free radical generation [40]. Significant increase in the MDA levels of goat sperm were observed with the treatment of piperine at 40 µmol/L, 60 µmol/L, 80 µmol/L, 100 µmol/L. From the above results, we may conclude that anti-fertility activity of the piperine could be due to the increased induction of OS.

CONCLUSION

Piperine decreases the activity of the antioxidant enzymes and hampers the epididymal environment where the sperm maturation takes place. Considering the above results, we propose that the inhibition of antioxidant enzyme activities along with a significant increase in LPO levels could generate ROS in the epididymis, which might be the reason for the potential antifertility effects of piperine. This determines that it could pave the way for the development of male contraceptives and also acts as a spermicide in female contraception. Further investigation is needed to find out the interaction between piperine and sperm maturation at molecular level.

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