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Beneficial Role of Ginger Powder (*Zingiber officinale*) against Acephate-induced Reprotoxicity in Adult Male Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study was aimed to investigate the protective role of ginger against acephate-induced testicular toxicity in adult rats.
Methodology: Rats were allocated into four groups where animals in group I served as controls, while animals in group II, III and group IV were treated as experimental rats. Rats in groups II, III and IV were treated with acephate (50mg/kg body weight), ginger (100mg/kg body weight) and combination of both acephate and ginger, respectively over a period of 60 days. After completion of experimental period sperm count, sperm viability, sperm motility, sperm membrane integrity, testicular steroidogenic marker enzymes (3β-HSD and 17β-HSD, serum testosterone and testicular architecture was performed in both control and experimental rats.
Results: Relative weights of reproductive organs, sperm count, sperm viability, sperm motility and sperm membrane integrity were significantly decreased in acephate treated rats over controls. Acephate administration also reduced the circulatory levels of testosterone associated with a significant reduction in the testicular steroidogenic marker enzymes (3β-HSD and 17β-HSD) in rats.

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The testicular architecture was disrupted in acephate intoxicated rats. In contrast, ginger administration significantly recovered the acephate-induced suppressed selected reproductive parameters with increased circulatory levels of testosterone and restoration of sperm endpoints in as compared to acephate alone treated rats. No significant changes were observed in any of the selected reproductive endpoints in ginger treated rats as compared to controls.

Conclusion: The results can be concluded that supplementation of ginger mitigates the negative effects of acephate on male reproductive health via amelioration of testicular setroidogenesis and spermatogenesis and epididymal sperm maturation events in rats.

Keywords: Acephate; ginger; sperm parameters; testosterone; steroidogenic enzymes.

1. INTRODUCTION

Nowadays there is a major concern towards the infertility related problems in populations at reproductive age. Several studies have shown that environmental toxicants underlie one of the major route causes for male infertility [1-3]. However, the exact mechanisms are not clarified. Among broad spectrum of environmental pollutants, the adverse effects of organophosphates (OPs) on humans and wildlife are alarming [4-6]. OPs are esters of phosphoric and thiophosphoric acids and their basic mode of action are linked to their ability to inhibit action of acetylcholinesterase [7]. During the past two decades, the endocrine disrupting properties of OPs have been explored and many studies have shown that OPs disrupt the hormone-dependent physiological functions including male reproduction [8-10]. Acephate is one of the widely used insecticides to protect agricultural crops, horticulture, viticulture in a variety of fields, fruits and aphids in vegetables. It is estimated that every year almost millions of Kilograms of acephate have been released into the environment, which in turn cause acephate accumulation in the food items [11]. The mode of action of acephate is believed to be inhibition of neurotransmitter, acetylcholinesterase (AchE) [12]. Many studies in experimental animals have shown that acephate is not target specific and thus, may be harmful to humans and wild life following exposure to acephate [13]. In congruent to this notion, it has been shown that acephate exposure leads to neurotoxicity, mutagenicity, carcinogenicity, and cytotoxicity in rodent models [14-16]. With regard to male reproduction, it has been shown that acephate exposure leads to reduction in testis weights, deterioration of sperm motility and its density, inhibition of testosterone synthesis from the testicular Leydig cells [13, 17-21].

In the current scenario, neutraceuticals that can able to protect the male reproductive health against environmental toxicants is one of the major areas of Ethnopharmacology [22-24]. The rhizome of ginger plant (*Zingiber officinale*) is one of the widely used food commodities all over the world. The pharmacological properties of ginger are well acknowledged [25-27]. Supplementation of ginger in diet offers many health benefits including antioxidant, anti-platelet, anti-tumor, antidiabetic, anti-rhinoviral, anti-hepatotoxicity, and anti-arthritic effects [28-30]. The positive effect of ginger on male reproductive health is well established is well appreciated [31, 32]. The steroidogenic properties of ginger were reported in male rats [29]. Gingers protective role has also been studied in rats treated with various testicular toxins such as cisplatin [33], cyclophosphamide [34], sodium arsenate [35], gentamicin [31] and lead [36].

Considering the facts that a) ginger with its antioxidant and steroidogenic properties promotes spermatogenic and testosterone biosynthesis and protects testis against oxidative injury and b) acephate-induced testicular toxicity occurs via oxidative stress and inhibition of testosterone synthesis from the Leydig cells and c) it is conceivable that the supplementation of ginger protects testis and its functions against acephate-induced oxidative toxicity. To accomplish this rationale, the probable effect of ginger on male reproductive health in rats subjected to acephate exposure was studied. The results indicated that supplementation of ginger protects testicular functions in acephate treated rats.

2. MATERIALS AND METHODS

2.1 Procurement and Maintenance of Experimental Animals

The test animals, healthy rats of Wistar strain were purchased from an authorized vendor (M/S Raghavendra Enterprises, Bangalore, India). The

rats after their arrival were maintained in polypropylene cages (18" x 10" x 8") containing sterilized paddy husk as bedding material. All the rats during acclimatization period were provided with standard rat chow and water *ad libitum*. The rats were maintained under controlled laboratory conditions such as air conditioned room maintaining a temperature between 24°C to 27°C with a 12-hour light and 12-hour dark cycle..

2.2 Chemicals

Acephate (Chemical name- O, S-dimethyl acetylphosphoramidothioate; Trade name- Orthene) (Image) purchased in a local pesticide shop, (Sri Venkateswara Pesticides) and dried ginger powder were purchased from Narasimha Ayurvedics respectively. The other chemicals were purchased from Himedia Pvt. Ltd.

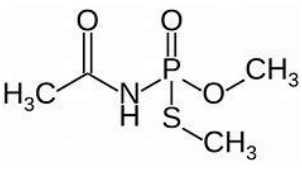

Image 1. Structure of acephate

2.3 Experimental Design

Male rats (90 days old) were allocated into four groups (n=8 per group). Animals in group 1 were considered as controls and they received only drinking water. Rats in group 2 and 3 received acephate orally (50 mg/Kg body weight) in drinking water and ginger via gavage (100 mg/Kg body weight in drinking water respectively over a period of 60 days, whereas rats in group 4 received combination of both acephate (50 mg/Kg body weight) and ginger (100 mg/Kg body weight) over a period of 60 days. In order to evaluate the toxic effect of acephate on spermatogenesis, a time line of 60 days was selected because in rats, completion of one spermatogenic cycle requires approximately 60 days [37-38]. The doses selected for acephate and ginger were based on previous. After completion of 60 days, the rats from control and experimental groups were sacrificed by cervical dislocation and the selected reproductive endpoints were determined. The organs were immediately isolated and weighed to its nearest milligram using Shimadzu balance. The relative weights of the reproductive organs were determined by using the formula:

Tissue somatic index (TSI) = weight of the organ/total weight of the animal x 100

2.4 Sperm Parameters

Epididymis tissue was used to evaluate the sperm endpoints. Briefly, the epididymal secretions obtained in physiological saline was used to determine the sperm count [39], sperm motility [39], sperm viability [40] and sperm membrane integrity using hypo-osmotic swelling test (HOS-T) [41]. Sperm count was determined using Neubauer haemocytometer and the sperm concentration refers to the number of spermatozoa per ml epididymal plasma and were expressed as millions/ml. The percent motility was determined by counting motile/non motile sperms observing under the Olympus microscope (model no.CX41). Tryphan blue (1%) staining method was used to determine the ratio of live to dead spermatozoa and the viable sperm was expressed in terms of percentage. Sperm membrane integrity was determined using HOS-test, wherein intact sperm when subjected to hypo-osmotic medium, due to influx of the medium, the tail coils due to hypo-osmotic stress. The percentage of coiled tails was calculated with the help of the Olympus microscope (model no. CX41).

2.5 Assay of Testicular Steroidogenic Enzyme Activities

The activity levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were estimated in the microsomal fraction of testis [42]. Briefly, the testicular tissue was homogenized in ice-cold 20 mM Tris HCl buffer (pH 8.2) and the microsomal fraction (enzyme source) was separated using centrifugation. The absorbance at 340 nm was measured at 20 sec intervals for 3 min in a UV-VIS spectrophotometer (Hitachi model, U-2001) against the reagent blank containing all the components, except the enzyme source. The enzyme activities were expressed in µ moles of NAD converted to NADH/mg protein/min (3β-HSD) and µ moles of NADPH converted to NADP/mg protein/min (17β-HSD). For the analysis of enzymes, zero order kinetics was followed. Preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration was also performed and recorded.

2.6 Histological Analysis of Testis

Immediately after isolation, Testes were cleared from the adhering tissues and fixed individually in Bouin's fixative for 24 h. The fixed specimens were dehydrated in ascending alcoholic series and after clearing in xylol, embedded in paraffin wax. Sections at 6 µm thickness were made. The sections were stained with hematoxylin followed by eosin [43] and examined under Olyrnpus microscope (Model no: BX41TF). Photomicrographs were taken using Olympus microscope (Model No: BX4 1 TF).

2.7 Assay of Serum Testosterone

Blood sample was collected from the heart of each animal prior to necropsy. The serum was separated by centrifugation at 2,000 X g for 5 min after overnight storage at 4ºC and stored at -80ºC until assayed. Radio immune assay (RIA) for serum testosterone was analysed according to method described by Rao *et al*. [44].

2.8 Statistical Analysis

Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The data were presented as mean ± SD and values $p<0.05$ were considered significant. All statistical tests were performed using Statistical Package for Social Sciences (SPSS), version 16.0.

3. RESULTS

During the experimental period, none of the animals from control and experimental groups were excluded from the present study. No clinical signs of toxicity such as lethargic movements, salivation, and loss of hair were noticed in acephate treated groups. No mortality was observed in any of control and experimental groups.

The relative weights of testis and epididymis were significantly decreased in acephate treated rats over controls (Table 1). On the other hand, the relative weights of reproductive organs were significantly increased in acephate plus ginger treated rats over acephate treated rats alone. Supplementation of ginger did not show any effect on the relative weights of reproductive organs in adult male rats as compared to controls (Table 1).

Analysis of sperm endpoints revealed that the acephate intoxication deteriorated sperm variables as evidenced by a significant reduction in the sperm count (Fig. 1A), sperm motility (Fig. 1B), sperm viability (Fig. 1C), and number of tail coiled sperm (Fig. 1D), in rats over controls. On the other hand, co-treatment of ginger and acephate showed a marked increase in the selected sperm variables in rats over its respective controls. No significant differences were observed in sperm endpoints between ginger supplemented rats and controls (Figs. 1-4).

The testicular 3β-HSD and 17β-HSD activity levels were significantly decreased in acephate treated rats over control rats. However, rats treated with both acephate and ginger showed a significant increase in the levels of testicular steroidogenase enzymes as compared to acephate treated rats. No significant changes were observed in the activity levels of testicular steroidogenic enzymes in ginger supplemented rats over controls (Table 2). Similar trend was observed with the testosterone levels. Acephate showed a reduction in the circulatory levels of testosterone in rats over controls, while ginger supplementation ameliorated the serum testosterone levels in acephate plus ginger treated rats as compared to its respective controls. However, no significant changes were observed in the serum testosterone levels in ginger alone administered rats over controls (Table 2).

Table 1. Effect of ginger (G) powder on the organ indices of acephate (ACE) treated rats

Parameter	Control	ACE	G	ACE + G
Testes	1.86 ± 0.06	1.14 * ± 0.03 (-38.70)	1.89 ^{ns} ± 0.07 (1.61)	1.79 * ±0.05 (57.01)
Epididymis	0.70 ± 0.03	0.52 * ± 0.02 (-25.40)	0.72 ^{ns} ± 0.06 (2.85)	0.67 * ± 0.01 (28.84)

*Values are mean ± S.D. of eight rats; Values in parenthesis are percentage change from controls; Values with * differ significantly from control at p < 0.05; ns = non significant; For evaluation of 'p' and percent change, for Acephate treated and Ginger treated rats, untreated rats served as controls; For Acephate + Ginger, Acephate served as control*

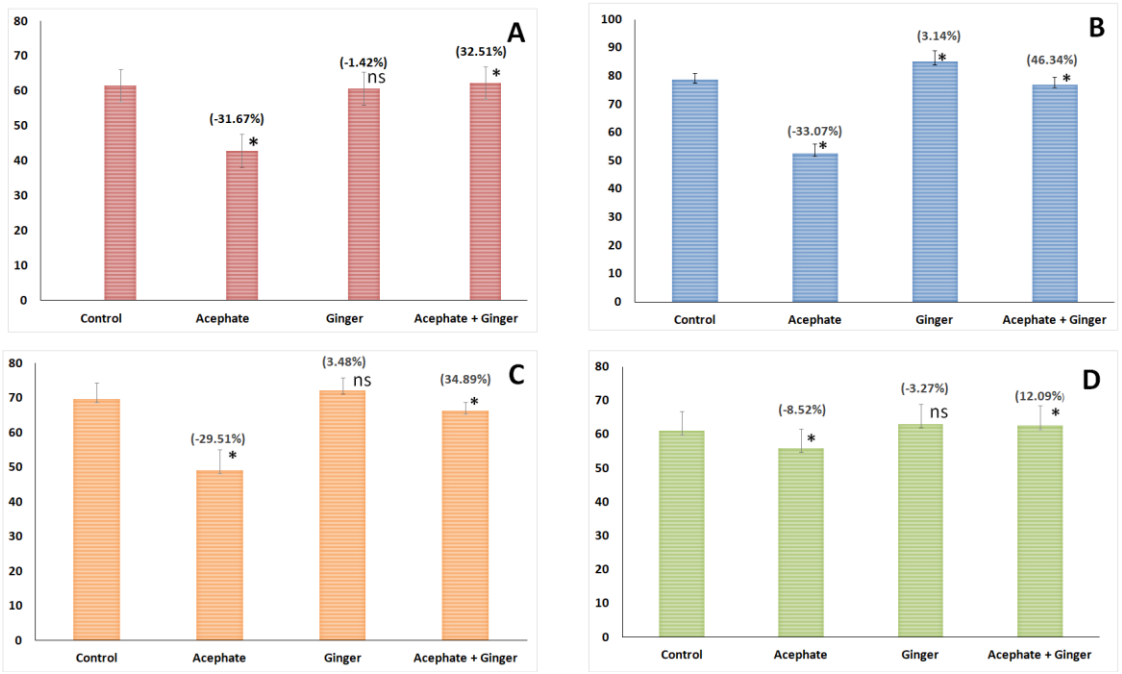

Fig. 1. Effect of ginger powder on selected sperm parameters of acephate treated rats; A: Sperm count; B: Sperm motility; C: Sperm viability; D: Number of tail coiled sperm

*Bars are mean ± S.D. of eight rats. Values in parenthesis on bars indicate percentage change from controls. Bars with * differ significantly from control at p < 0.05; ns = non significant. For evaluation of 'p' and percent change, for Acephate treated and Ginger treated rats, untreated rats served as controls; For Acephate + Ginger, Acephate served as control*

Table 2. Effect of ginger (G) powder on testicular 3β- Hydroxy steroid dehydrogenase and 17β-Hydroxy steroid dehydrogenase enzymes and serum Testosterone levels in acephate (ACE) treated rats

Parameter	Control	ACE	G	ACE + G
3β- HSD (µmoles of NADPH converted to NADP/mg protein/min)	0.89 ±0.12	0.61 * ±0.09 (-31.46)	0.92 ns ±0.41 (3.37)	0.87 * ±0.81 (42.62)
17β- HSD (µmoles of NADPH converted to NADP/mg protein/min)	0.44 ±0.04	0.32 * ±0.06 (-27.27)	0.46 ns ±0.04 (4.54)	0.42 * ±0.05 (31.25)
Testosterone (ng/ml)	7.54 ± 0.24	2.11 * ± 0.07 (-72.01)	7.23 ns ±0.35 (-14.6)	4.91 * ± 0.58 (-8.35)

*Values are mean ± S.D. of eight rats; Values in parenthesis are percentage change from controls; Values with * differ significantly from control at p < 0.05; ns = non significant; For evaluation of 'p' and percent change, for Acephate treated and Ginger treated rats, untreated rats served as controls; For Acephate + Ginger, Acephate served as control*

Testis of control rats showed apparently normal architecture of seminiferous tubules as indicated by epithelial layer as basement membrane, and also showing various stages of germ cells (Fig. 2A). The morphology of testis in acephate administered rats exhibited signs of disruption as evidenced by rupture in epithelium, and necrosis of spermatogenic cells. In addition, a marked reduction in the number of spermatocytes, spermatids, and spermatozoa were also observed in testis of acephate treated rats (Fig. 2B). Whereas restoration of testicular architecture accompanied by various stages of spermatogenic cells were observed in testis of rats co-treated with acephate plus ginger (Fig. 2C) as compared to acephate treated group. On the other hand, no significant changes were noticed in the testicular architecture of ginger treated rats over controls (Fig. 2D).

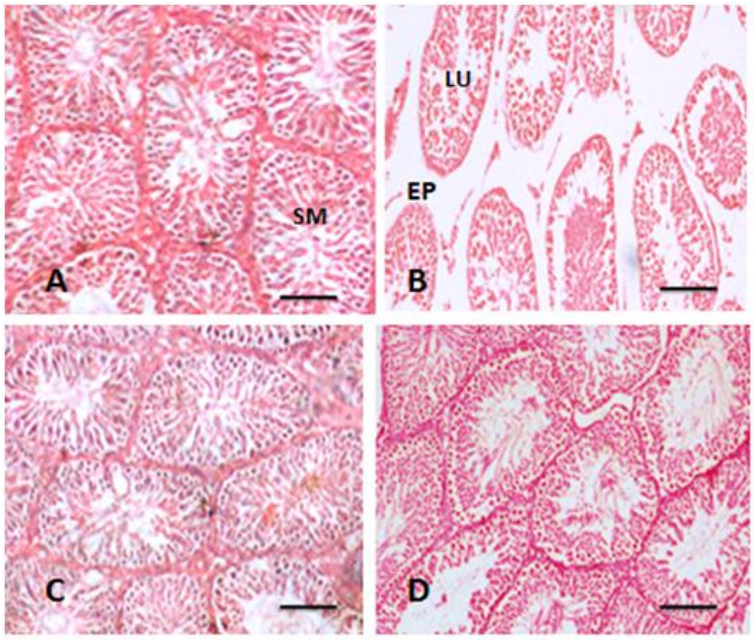

Fig. 2. Photographs testicular architecture of control rats (A) and acephate treated rats (B), Ginger (C) and Acephate + Ginger treated rats (D). SM = sperm; EP = epithelium; Lu = lumen;. Scale bar = 25 µm

4. DISCUSSION

The results indicated that supplementation of ginger restored male reproductive health in rats treated with acephate as indicated by a) increase in testis and accessory sex organ weights, b) enhanced epididymal sperm endpoints such as sperm motility, sperm viability and sperm membrane integrity, c) increase in circulatory levels of testosterone and d) recovery of testicular architecture.

Testis plays a key role as exocrine and endocrine organ of male reproductive tract [45]. It comprises of three major cells, the germ cells, the Sertoli cells and the Leydig cells. Among the testicular cells, the Sertoli cells sustain germ cells thereby promote spermatogenesis, while the Leydig cells play an essential role in testosterone biosynthesis [45]. Acephate exposure caused a reduction in testicular spermatogenesis and steroidogenesis in rats, suggesting that acephate intoxication can able to induce cellular toxicity at various levels of testicular cells, germ cells, the Sertoli cells and the Leydig cells. Furthermore, it has been shown that the testicular mass represents differentiated spermatogenic cells [46,47]. The disruption of testicular architecture in acephate treated rats might cause abnormal sperm production due to germ cell loss and thereby attributed to reduced testicular weight. Moreover, abnormal spermatogenesis could be attributed to the improper support to the germ cells by the Sertoli cells [48,49]. Previously, it has been shown that degeneration of germinal epithelium leads to reduced number of spermatogenic cells and consequently inhibits spermatogenesis [13]. Morphological alterations in testis associated with reduced spermatogenesis during OPs intoxication was previously reported [50]. In addition, acephate exposure over a period of 60 days caused a reduction in the weights of accessory sex organs in rats. This could be linked to the reduced bioavailability of testosterone in acephate treated rats [18,19] because the structural and functional integrity of male reproductive tract largely depends on adequate supply of androgens [51]. Thus, reduced reproductive organ weights, spermatogenesis and epididymal sperm maturation events in acephate treated rats could be associated with inadequate supply of androgens [13,18,19,21]. Accordingly, we found a significant reduction in the circulatory levels of testosterone in acephate treated rats. Testosterone biosynthesis depends on a cascade of enzymes including 3β-HSD and 17β-HSD [52] which are classically known as testicular steroidogenic marker enzymes. The findings of this study indicated that the activity of testicular steroidogenic marker enzymes showed

a marked reduction in acephate treated rats, suggesting that 3β-HSD and 17β-HSD might be susceptible targets during acephate exposure [21]. Our findings also support the findings of earlier studies [13,18,19] wherein these authors demonstrated that acephate exposure deteriorate testicular spermatogenesis and steroidogenesis in experimental animals. Based on the findings i.e. reduction in circulatory levels of testosterone associated with reduced activity levels of testicular steroidogenic marker enzymes, it could be hypothesized that acephate intoxication negatively targets biosynthetic machinery of Leydig cells in rats. This notion is supported by previous studies [21,53]. Previous studies have shown that OPs such as dimethoate can able to interfere with testosterone biosynthesis via disrupting StAR protein, steroidogenic acute regulatory protein which channels cholesterol (a precursor of testosterone) from outer to inner mitochondrial surface [53]. However recently, *in vitro* studies shown that acephate exhibits similar properties like dimethoate and thus down-regulate the expression of StAR, luteinizing hormone receptor and 3β-HSD in rat immature testicular Leydig cells [21].

One of the important findings of this study revealed that the supplementation of ginger powder (via gavage) ameliorated acephate induced reproductive toxicity in adult male rats. Significant increase in the weights of reproductive organs in acephate plus ginger treated rats might reflect steroidogenic properties of ginger [31,34-37,54]. Interestingly, earlier studies reported that supplementation of *Z. officinale* significantly increased weights of testes and epididymal α-glucosidase activity in rats [33]. Ginger supplementation significantly enhanced the sperm count suggesting amelioration of testicular spermatogenesis and significant increase in sperm viability, sperm motility and sperm membrane integrity suggesting amelioration of epididymal sperm maturation events in rats subjected to both acephate plus ginger in rats. Protective effects of ginger against a range of spermatotoxic agents was also previously reported [31, 33, 35]. Studies related to the effect of ginger on male reproduction in rodents have shown that the exogenous supplementation of ginger might be linked to the enhancement of sperm motility and its viability via promoting epididymal and vas deferens functions [30,35,54,55]. Though the exact mechanism of action of ginger on elevated serum testosterone levels associated with enhanced testicular spermatogenesis and sperm maturation events cannot be determined from the current study, based on the findings and also available information from the literature, we hypothesize the protective effect of ginger could underlie three potential mechanisms: ginger supplementation acts at two important levels, testosterone biosynthesis and elevation of antioxidants [56]. With regards to testosterone biosynthesis, andrological studies have shown that the supplementation of ginger elevated cholesterol levels which in turn acts as a seminal factor for the biosynthesis of testosterone [29] and further it has been shown that supplementation of ginger could act at the level of pituitary gland for the synthesis of luteinizing hormone which in turn triggers testosterone production from the Leydig cells of experimental models [30]. Surprisingly, *in vitro* studies demonstrated that acephate down regulate the expression of cholesterol channeling StAR protein, luteinizing hormone receptor and 3β-HSD in the Leydig cells isolated from immature rats [21]. Piecing the data, it might be assumed that the supplementation of ginger can stimulate Leydig cell steroidogenesis which consequently leads to enhanced circulatory levels of testosterone in rats co-administered with ginger and acephate. With regard to antioxidants, it has been shown that ginger root comprises of several bioactive compounds with antioxidant properties including gingerols, zingiberene, zingerone, glucosides-6-gingerdiol, and flavonoids [57]. Previously, it has been shown that acephate intoxication provokes oxidative stress in the testis of rats [20]. Thus, we propose that the antioxidant properties of ginger could protect the testis against acephate induced oxidative damage which in turn ameliorated testicular architecture in rats. On the other hand, with regards to spermatogenesis, several studies have shown that the supplementation of ginger promotes sperm production, prevents testicular apoptosis in experimental models [30,58].

5. CONCLUSION

Finally, based on the results it can be concluded that the supplementation of ginger mediated amelioration of reproductive organ weights, spermatogenesis, and sperm maturation events could be attributed to the maintenance of adequate bioavailability of testosterone in rats co-administered with acephate and ginger. Further, studies are in progress to elucidate the molecular mechanisms that underlie ginger

mediated protection against acephate induced testicular toxicity in rats.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals [37] Government of India and approved by the Institutional Animal Ethical Committee, SPMVV, Tirupati

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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