ROLE OF DIOSGENIN ON SPERMATOGENESIS AND STEROIDOGENESIS IN SWISS ALBINO MICE

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ROLE OF DIOSGENIN ON SPERMATOGENESIS AND STEROIDOGENESIS IN SWISS ALBINO MICE

BY

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Department of Zoology

Submitted in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

CERTIFICATE

I certify that the thesis entitled "**Role of Diosgenin on Spermatogenesis and Steroidogenesis in Swiss Albino Mice**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **KHUSHBOO MAURYA** is a record of research work carried out during the period from 2015-2018 under my guidance and supervision and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this university or any other university or institution of higher learning.

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ACKNOWLEDGEMENT

The work presented in this thesis would have not been possible without my close association with many people. I take this opportunity to extend my sincere gratitude and appreciation to all those who made this Ph.D. thesis possible.

First and foremost, I would like to extend my sincere gratitude to my research supervisor **Prof. G. Gurusubramanian**, Department of Zoology, Mizoram University, who gave me his precious ideas to do this wonderful work. I shall always be obliged for his dedicated help, advice, inspiration, encouragement and continuous support, throughout my Ph.D. Working with him, I came to know about so many new things and ideas to explore new areas of research. He always introduced me to this exciting field of science. His enthusiasm, integral view on research and his mission for providing high-quality work, has made a deep impression on me. During the discussion of our lab work, I have learnt extensively from him, including how to raise new possibilities, how to regard an old question from a new perspective, how to approach a problem by systematic thinking, data-driven decision making and exploiting serendipity.

I express my heart-felt gratitude to **Dr. Vikas Kumar Roy,** Assistant Professor, Department of Zoology for his continued support and encouragement as well as guidance. His scientific inputs, personal helps and friendly nature has always made me feel at ease with him and I could always look back on him for any support whenever I needed throughout my Ph.D. He always helped me out when I got any difficulty or query regarding experiments.

I am grateful to **Prof. G.S. Solanki, Prof. G.C Jagetia**, **Dr. Amit Trivedi, Dr. H.T. Lalremsanga, Dr. Zothansiama, Ms. Esther Lalhminghani (**faculty of department of Zoology, Mizoram University, Aizawl) for their guidance and moral support. I am also thankful to the non-teaching **staff of department of Zoology**, Mizoram University, Aizawl for their valuable help in official matters.

I would like to give my deepest thanks to my parents and family members, who have given me emotional support throughout my Ph.D. I express my heart-felt gratitude to my in-laws family, specially my father in-law and mother in-law for their moral as well as physical support to finish my lab work and thesis writing.

It will be very less to express thanks to my husband, **Amish Verma**, who not only encouraged me to do Ph.D., but always stands behind me for any kind of help. I always use to get surprised with him when he solves my problems and confusions related to my Ph.D. He stands with me always even in worst situations. Without his love, care, and patience, it was impossible for me to continue my Ph.D. He will always be the role model for me. Thanks once again!

My cute **Aayushman's** smile makes me energetic. His naughty behavior was enough to remove my stress throughout the lab work as well as during thesis writing. Your presence provides me extra strength and power to work. Thank you my baby!

It will be really less to express my thanks to **Sunita Maibam Devi, Sanasam sanjeev, Meesala Krishna Murthy, Sagun**, **Lalrinzuali, Bidanchi R. Momin, Pratima** and **Indira sonar** for their continuous support, encouragement and for facilitating with all the requirements during my Ph.D. work.

Last but not the least, I would like to thank rest of my friends as well as my neighbours for their love and support.

Khushboo Maurya

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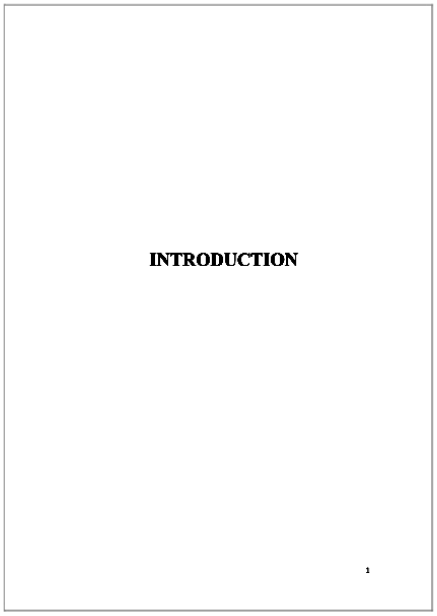

INTRODUCTION

1

Khushboo Maurya Thesis

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PRIMARY SOURCES

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2	Sumit Sethi, Chandra Mohini Chaturvedi. "Temporal synergism of neurotransmitters (serotonin and dopamine) affects testicular development in mice", Zoology, 2009 Publication	<%1
3	Mounir Ouzir, Khalid El Bairi, Saaïd Amzazi. "Toxicological properties of fenugreek (Trigonella foenum graecum)", Food and Chemical Toxicology, 2016 Publication	<%1
4	Vikas Kumar Roy, Lalramdinthara Chenkual, Guruswami Gurusubramanian. "Protection of testis through antioxidant action of Mallotus roxburghianus in alloxan-induced diabetic rat model", Journal of Ethnopharmacology, 2015 Publication	<%1
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LIST OF ACRONYMS

%	percentage
°C Degree	Celsius
3β-HSD	3β-hydroxysteroid dehydrogenase
µg	Microgram
µL	Microlitre
ADR	adriamycin
ALP	Alkaline Phosphatase
b.w.	Body weight
CDNB	1-chloro-2,4-dinitrobenzene
D	Diosgenin
DBP	di-n-butyl phthalate
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
DTNB	Dinitrobenzene
DSP	Daily sperm production
DTNB	5,5 dithio 2-nitobenzoic acid (Dithionitrobenzene)
E2	Estradiol
ER	Estrogen receptors
ELISA	Enzyme linked immuno sorbant assay
FSH	Follicle stimulating hormone
g	Gram
GC-MS	Gas chromatography mass spectrometry
GPCRs	G protein coupled receptors
GSH	Glutathione reduced
GST	glutathione S–transferase
hCG	human chorionic gonadotrophins
HDL	High Density Lipoprotein
H_2O_2	Hydrogen peroxide

HSD	Hydroxysteroid dehydrogenase
JTBS	Johnsen's testicular biopsy score
kg	Kilogram
L	Litre
LDH	Lactate dehydrogenase
LHR	Luteinizing receptors
MDA	Malondialdehyde
mg	Miligram
mL	Mililitre
MSTD	Mean seminiferous tubule diameter
NBT	Nitrobluetetrzolium
ND	Nandrolone decanoate
ng	Nanogram
OECD	Organization for Economic Co-operation and Development
PCNA	Proliferating cell nuclear antigen
PUFA	polyunsaturated fatty acid
ROS	Reactive oxygen species
s	Second
SGPT	serum glutamic-pyruvic transaminase
StAR	Steroidogenic acute regulatory protien
TBARS	Thiobarbituric acid reactive substances
U	Unit
WHO	World Health Organisation
wt	Weight
ZP	Zona pellucida

INTRODUCTION

1.1. Introduction

Diosgenin (22alpha-Spirost-5-en-3beta-ol), known as the "pro sex hormone" due to its steroid nature. Recently, the production of synthetic hormone like testosterone, estradiol and progesterone has been increases for different pharmacological purpose (Chen *et al.*, 2015). In pharmacological industry, the highest levels of diosgenin are obtained from *Trigonella foenum, Costus speciosus, Smilax,* and many species of *Dioscorea* (Chen *et al.,* 2011). Diosgenin, undoubtedly, one of the most potent antioxidant have been described as free radical scavenger, has been rapidly increasing demands for various anti-inflammatory, anti-proliferative and apoptotic properties during the last decades. Beside of that, diosgenin has an important role in glucose and fat metabolism (Son *et al.,* 2007; McAnuff *et al.,* 2005). Several other function have been reported like anticancer effects in proliferating cancerous cell (Das *et al.,* 2012), anti-bone loss in estrogen deficiency (Chiang *et al.,* 2011), anti – depressive (Ho *et al.,* 2012), anti- osteoporosis (Chen *et al.,* 2015) and anti-ageing effect (Ho *et al.,* 2012). Most interestingly, several researchers reported diosgenin as estrogenic agent (Aradhana *et al.,* 1992; Accatino *et al.,* 1998) because its structure shows similarity with estrogen as well as progesterone (Higdon *et al.,* 2001; Au *et al.,* 2004). However, recently, Medigović *et al.* (2014), reported diosgenin did not act as estrogenic agent in immature female rat.

It is already well known and reported in various researches, phytoestrogen having structure similarity with several steroids like cortical, estradiol and progesterone, may interferes in process of steroid production (Kuiper *et al.,* 1998). Additionally, there is recent trend with steroid exposure in various disorders to

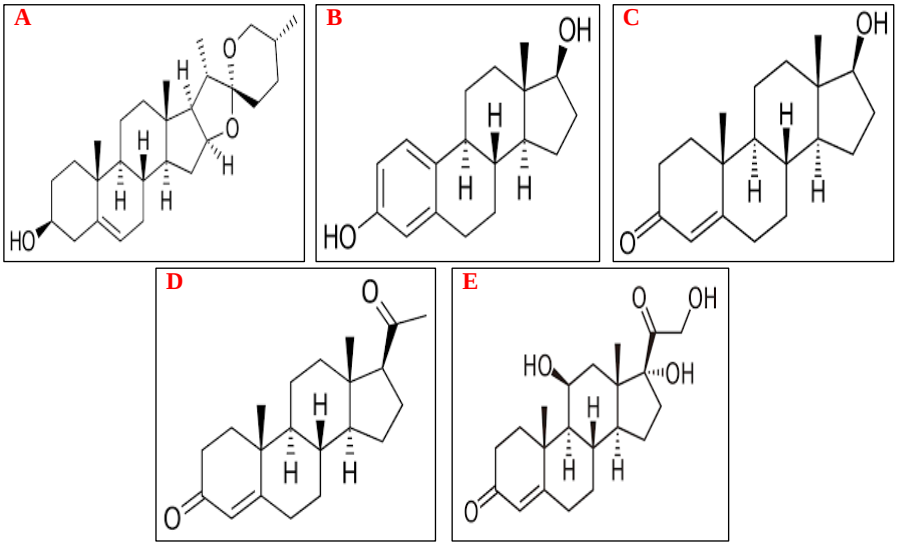
ignore menopausal symptoms, used as anti-ageing, anti-arthritis, anti-osteoporosis and to cure hormonal misbalance in various syndrome of metabolism. There is many evidences suggested steroid saponin present in phytosteroid, are major factor involved for their estrogenic or anti-estrogenic effect.

Recently, phytosteroid also known as dietary estrogen has shown to interact and interfere with endogenous hormone thus affecting the normal physiology and metabolism activity (Degen and Bolt, 2000). Steroid derived from plant source can mimics and interacts with estrogen receptor thus affecting the endocrine hormone function.In addition to that, an exogenous steroid may act as endocrine disruptor by modulating the endogenous sex hormone ratio (Safe, 2001) .Moreover, notably, due to potential risk of endocrine dysfunction with phytosteroid, it is not considered as safe. In a recent study by Medigović *et al.* (2012) investigated that genistein, a phytosteroid, considered as anti – folliculogenesisin female as it arrested the development of initial follicle. Another research by Skakkebaek *et al.* (2001) reported phytoestrogen as anti-androgen due to adverse effect on reproductive physiology and alteration in sperm formation. Still, it is matter of debate, whether phytosteroid directly interfere in pituitary gonadal axis or alter the steroid receptor function due to its binding ability with estrogen receptors (ER). However, there may be another possibility of reproductive dysfunction with phytoestrogen exposure, is raised oxidative stress, considered one of the most important factor that generate excessive production of reactive oxygen species (ROS). Based on the previous and recent literature data, we hypothesized that the diosgenin, a potent new synthetic steroid precursor agent, widely used in pharmacological and industrial purpose; may

interfere in testicular function in normal healthy mice. However, to our knowledge, diosgenin exposure and related reproductive toxicity in male mice has not been studied till date. Interestingly, researched done by Medigović *et al.* (2014), revealed diosgenin does not act as estrogenic agent in immature rat model of an uterotrophic assay, has opened a new way of our research in male reproductive system.

Diosgenin is an aglycon, structurally similar to cholesterol and principal raw material for the industrial production of steroid drugs (**Fig. 1**). *Trigonella*, *Costus* and *Dioscorea* spp. are reported as the main source of its active compound- diosgenin. Diosgenin, as an active compound reported in above said species are used for many therapeutic purposes, against anorexia, diabetes, jaundice, gastritis and many others which are not well established by the previous literature. Though, diosgenin is a major active constituent of above said species, and plant extract have been shown to affect male and female reproductive functions thus there is need of caution during use of fenugreek for therapeutic purpose.

Researchers have paid great attention to steroid like compounds present in numerous dietary agents because of associated reproductive risks due to the side effect of herbal plants. While good deals of research have been conducted on the reproductive toxicity of these plants, there is a lack of information about the mechanisms involved in the pathogenesis of male reproductive dysfunction induced by diosgenin**.**



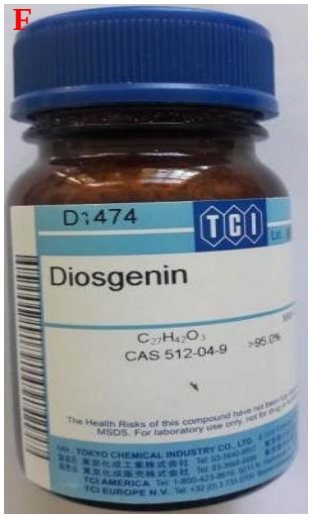


Fig. 1. Structure of Diosgenin **(A),** Estradiol **(B),** Testosterone **(C),** Progesterone **(D),** Corticosteroid **(E)** and Diosgenin Drug **(F).**

1.2. General description of diosgenin

Diosgenin is an aglycon and belongs to a class of compound called as triterpenoid, which are tremendously weak acidic compound.

Molecular Weight	:	414.63 g/mol
Molecular formula	:	$C_{27}H_{42}O_3$
Chemical nature	:	Steroid
Storage temperature	:	2-8 °C
Melting point	:	204 - 207°
Optical Rotation	:	(a) 25D – 129 (c, 1.4 in CHCl3)

1.3. Dietary and pharmacological sources of diosgenin

In *Dioscorea* and *Costus speciosus* species, rhizomes are the major source of diosgenin while in fenugreek well known in seed. The most common *Dioscorea species* are *D. bulbifera L.* (Pita aalu), *D. pentaphylla L.* (Panja Sanga), *D. hispida Dennst.* (Banya aalu), *D. alata L.* (Khamba aalu), *D.oppositifolia L.* (Paani aalu) and *D. pubera Blume (*Kukai Sanga) etc. The rhizome and seed of plants are extracted in laboratories for making various kinds of steroids, is often used as "natural alterative" for steroid therapy in case of ageing disorder (osteoporosis, reproductive dysfunction and poor sex desire) and hypogonadism in some cases of metabolic syndrome in both male and female. Diosgenin content up to 3.37% has been reported in rhizome of *C. speciosus* (Singh *et al.,* 2013), in commercial species of *Dioscorea*, it contains 2 –

8% on dry weight basis (Kunjithapadam, 1977) and diosgenin present in fenugreek seed is reported around 0.2 - 0.9 % (Taylor *et al.,* 2000).

1.4. Role of phytosteroid (diosgenin) content on reproductive physiology

Traditional herbal medicines have demonstrated a bright future in discovering of new drug and to understand the importance of traditional herbsover all worldwide. But there is urgent need of awareness on herbal medicine due to their various kind of phytochemical constituent (steroidal compound, diosgenin) as it may inhibit individual fertility potential. Without proper investigation, people commonly consume herbal medicine on daily basis to get their nutritional and medicinal requirements for being fit and healthy. According to McGuffin *et al.* (1997), he defined safety of use of herbal drug as "Herbs that can be used safely when used appropriately". Many of traditionally used herbal drugs have been used to treat various kinds of diseases and disorders. Recently, there is increased trend in pharmacology to study antifertility agents in known herbal plant due to lack of information related to reproductive toxicity.

Fenugreek is one of the herbal medicines and well-studied for its associated reproductive risk. Both seed and leaf of fenugreek is traditional herbs in India and many other countries, used to treat various disorder of metabolism, such as lowering of blood sugar level, to decrease serum bad cholesterol, to relieve in pain of joints and for good digestion. Besides that, it has been traditionally used as to reduce menstrual pain, for ageing disorder or post-menopausal problems, for labor pain during delivery, for lactation and as contraceptive. Probably, pain relieving effect is

due to presence of its steroid saponin, diosgenin. Additionally, co-administration of fenugreek extract or as whole seed caused significant decrease in male and female reproductive parameters as well as fertility potential. Notably, foetal toxicity and teratogenicity are also a concerned issue about fenugreek consumption.

Dioscorea, well known as yam, contains a steroid saponin (diosgenin) used in laboratory for various kind of steroid production, such as estrogen and dehydroepiandrosterone (DHEA).Recently, it has been investigated that 1 g *Dioscorea* contains about 9.5 mg of diosgenin (Sato *et al.*, 2007) which is saponin known for reproductive impairment. *Dioscorea* contains saponin (diosgenin), caused reproductive toxicity. Work reported by Mohan *et al.* (2011), revealed that there is dose dependent effect of *Dioscorea* on reproductive system. According to him, ethanol extract of *D. esculenta* significantly inhibited all sperm parameters along with marked decreased serum testosterone, which is crucial factor for male fertility. Another report from Das *et al.* (2014), *D. bulbifera* plant paste is traditionally used by female for contraceptive as well abortificient activity. However, further research is needed to prove whether the effect is due to presence of its bioactive compound saponin (diosgenin) and changes are reversible or permanent.

C.speciosus, known as crepe zinger for its ethno- medicinal use and diosgenin is main active constituent of this plant. Though, presence of saponin (diosgenin) in *Costus* is extensively used in the various kind of clinical treatment of metabolic disorder and for anti -proliferation purpose. However, there is insufficient report on its reproductive associated risk and related mechanism behind reproductive toxicity. Diosgenin may interfere in steroidogenesis process along with several adverse

effects, including reproductive toxicity. Diosgenin is the major active phyto-constituent present in *C. speciosus*. Tewari *et al.* (1973) revealed that *C. speciosus* exhibited an anti-fertility effect in male mice through a significant reduction in both spermatozoa level and quality. He found diosgenin isolated from *C. speciosus* was potent estrogen because 1600 µg diosgenin (I) [512-04-9] activity was approx. equal to a well-known estrogenic drug neoclinestrol (150 µg). One another report from Singh *et al.* (1972), claimed for its estrogenic activity from saponin isolated from the rhizome of *C. speciosus* causes increased uterine weight with some morphological changes along with increased glycogen content of uterine endometrial. Choudhury *et al.* (2012), revealed *C. speciosus* as ethno-medicine for local people used as fertility control due to presence of naturally active estrogen activity. Most recently, Das *et al.* (2014) investigated the paste of mature seed of *C. speciosus* is traditionally used as abortificient purpose when consumed for 3 days. Finally, concluding it all, phytosteroid (diosgenin) seems to have major role in reproductive physiology.

1.5. Relation between phytosteroid and fertility

A great variety of phytosteroid are abundantly found in many known ethanoherbal medicine, which have important role in plant cell structure and for production of growth hormone. These phytosteroids have been traditionally used as folk medicine. There are various kinds of phytosteroid naturally found in many herbs interfere in fertility process via disrupting the endocrine hormone function (Bennetts *et al.,* 1946; De Lange, 1961). Phytosteroid known for mainly estrogenic effect however, may also response as phytoestrogen, phytoandrogene or phytocorticoids. However, it is well known that phytoestrogen can also trigger their action as

phytoandrogenic effect. First of all, phytosteroid were reported by Bennetts *et al.* (1946), in sheep grazing on subterranean clover in western Australia reduces fertility potential. Notably, enzyme involved in steroidogenesis is directly affected by phytosteroid either by activation of estrogenic receptor or by androgen receptor. However, impact of phytosteroid on reproductive physiology and their influence on fertility potential a matter of debate whether steroid receptor coupling is altered by impaired endocrine function. Various steroid receptor and role of phytosteroid responsible for altered steroid receptor signaling pathway have been a new sight for novel research to characterize and determined their involvement in steroid metabolism and physiology. Recently, phytosteroid present in *Chehelghoza spp.*, significantly reduces the sperm parameter when allowed in different percentage in their daily food for 14 days (Safari *et al.*, 2017*)*. It's very interesting to know that evidence shows reduced fertility is also dependent on phytosteroid metabolites and their interference in steroidogenesis. Very interestingly, diosgenin, an example of phytosteroid, derive their name from steroid isolated from *Dioscorea*. Diosgenin, as, valuable drugs used in pharmacology has been made a major concern about its use. First of all, Stoll *et al.* (1952) reported that steroid extracted from *Digitalis lanata* was used for myocardial dysfunction by the Scottish physician William Withering (1785).

1.6. Cholesterol in reproductive physiology

Cholesterol plays a major role in animal physiology due to precursor of many steroid hormones (Gross *et al.*, 1971). It is one of the crucial factors for maintenance of metabolic function of body. Steroid can be categorized as corticoid (produced in

adrenal cortex) and sex steroid (produced in gonads and placenta) responsible for several physiological process involved in glucose metabolism, mineral or ion metabolism and for sexual characteristics development. Besides that, steroid known for their anti-inflammatory, immune booster, in case of osteoporosis, ageing disorder, wound healing etc. The process by which reproductive hormone produced in gonads and adrenal are known as steroidogenesis. Testis, ovary and placenta are well defined for steroid hormone production. Adrenal is another site of steroidogenesis, also produces the mineralocorticoids (MC) and glucocorticoids (GC) in zona glomerulosa and zona fasiculata respectively. Hypothalamus and pituitary glands are well connected with gonads and other organs in order to control the hormone secretion. The hypothalamic-pituitary-gonadal axis plays an important role in maintaining the steroid homeostasis by both negative and positive feedback mechanism (Gilbert, 2010). Excess production of cholesterol is associated with heart complication as well several kinds of endocrine disorder leading into syndrome. Any alteration in process of steroidogenesis, either in gonads or other parts of body, leading into reproductive impairment followed with infertility.

1.7. Reproductive toxicity and its assessments

Reproductive toxicity is severe condition of reproductive alterations that further affect the fertility potential of an individual. There are various kinds of environmental factors and industrial byproduct has been reported as endocrine disrupters that can manifest adverse effect at gestational as well puberty (Lewis, 1991). Reproductive toxicants such as certain drugs, phthalate, pesticides, heavy

metals, PCBs, glycol ethers, synthetic steroid with estrogenic or anti estrogenic activity have been shown a negative effect on gonadal function and suppress the production of gametes (Thomas, 1981; Colborn *et al.*, 1993). Reproductive toxicity due to any chemical or drug exposure not only affects the gametogenesis, it also modifies the behavioral nature of individual. There are various mode of drug toxicity such as suppression of biosynthesis of amino acids and cell cycle arrest at meiotic as well mitosis level leading into failure of gonads function and loss of embryo at gestational level (McLachlan, 1980). In adult individuals, the exposure of drug disrupts the production and efficacy of gametes as well growth of embryo. Drug with estrogenic or anti-estrogenic activity, can modify or harm the development of reproductive tract during gestational period. Gonads are extremely sensitive with exogenous steroid and xenobiotic agents, thus gonadal enzyme activity can be interrupted by metabolites of exposed drugs.

In male, it is previously reported that therapeutic drugs with estrogenic effect has been shown as suppression of testicular function. Low testis weight due to exposure of reproductive toxicants is a precise indicator of possible testicular tissue degeneration especially at higher doses (Ku *et al.*, 1993). Leydig cell and Sertoli cells, necessary for steroidogenesis, are susceptible to exogenous steroid and toxicants due to increase in inflammation and apoptosis. Several other such as testicular atrophy due to poor blood supply to germinal epithelium leading into to increase in germ cell loss and decline total in sperm mass. Seminiferous tubule with delaminating spermatogonia from its basal region, necrotic cells, multinucleated giant cells, vacuolated spermatocytes and low or less spermatids are documented as

sever reproductive toxicity in testis of male individual. Other accessory organs of testis are also androgen-dependent and exposure of toxicants may alter maturation of sperm. The loss of secretary function of epithelium of epididymis, seminal vesicle and prostate are known to affect progressive movement of sperm. Bioaccumulation of such toxicant also have been reported in testis as well in accessory sex organs lead into malfunction of spermatogenesis as shown by a noteworthy decrease in the number of different ages of germ cells at explicit stages in spermatogenesis cycle. Sperm motility is the most critical proportion of semen quality in reproductive assessment and can be a compensatory factor in male after toxicant exposure. Long term of toxicant exposure in male can alter the sperm morphology even in offspring (Wyrobek and Bruce, 1978) and can be a precise indicator of spermatotoxicity effect (Zenick *et al.*, 1994). It is well reported that alteration in sperm quality increases the disability of sperm to fertilize the egg (Nestor and Handel, 1984). Previously, drug such as cyclophosphamide, reported as genotoxic agents due to its DNA damaging property (Qiu *et al.,* 1995) can be easily inherited in their progeny (Hales *et al.,* 1992). Another more interesting fact about exposure of toxicants is failure of fertilization rate due to ability of such toxicants to reduced sexual behavior of male. Lack of adequate sexual behavior in male is necessary for preparation of female reproductive tract to transport the sperm to ovum (Adler and Toner, 1986).

Like male, female aspect of reproductive alteration is also modulated by endocrine function. Female shows a regular reproductive cycle, controlled by a specific endogenous hormonal balance. Exposure of toxicants can lead an imbalance of hormonal level that significantly alters the ovarian function. Decrease in ovarian

weight, depletion of folliculogenesis and its maturation is precise indication of ovarian toxicity (Kurman and Norris, 1978). Furthermore, failure of uterine endothelium to conceive a fertilized egg is one of symptoms of female reproductive toxicity. A number of endocrine disrupters present in environment such as pesticides, phenol derivatives, phthalates, halogenated compound and phytoestrogen are known to interfere in uterine as well ovarian ultrastructure and function (Kupfer, 1987; Hughes, 1988). The potential of ovary to produce the egg is dependent of gonadotrophins as well ovarian hormones (McNatty, 1979). Long duration of such environment toxicants known to delayed ovulation (Cooper *et al.,* 1994). Chemicals such as polyaromatic hydrocarbons, DDT, atrazine, dioxin and diethylstilbestrol (DES) etc are known to interferewith the hypothalamic pituitary-gonadal- axis leading into suppression of FSH, LH and gonadotrophins (Mattison and Nightingale, 1980; Cooper *et al.,* 1996; Giusti *et al.,* 1995; Harrison *et al.,* 1995). Furthermore, some chemicals such as carbendazim and ethylene oxide have been shown to inhibit the fertilization and zygote viability respectively (Zuelke and Perreault, 1995; Generoso *et al.,* 1987).

Another most susceptible form of reproductive toxicity in human is reported during prenatal and postnatal development. It can affect the viability percent, morphological deformity, metabolic syndrome and sterility. Exposure of toxicants can also alter the viability, weight gain, number and sex ratio among neonatal. Maternal exposure may bring out increase in gestational period, complications in parturition, dead fetus and premature delivery.

1.8. Enzymes involved in steroidogenesis

Steroidogenesis is complex process in which cholesterol is converted into potent androgens estrogens and progesterone through several catalytic and metabolic activities (**Fig. 2**). There are number of steroidogenic enzymes involved in production of several kinds of steroid hormone. The initial step of steroidogenesis is start with cholesterol transport by steroidogenic acute regulatory (StAR) protein to mitochondrial membrane in steroidogenic organs or tissue. Further, in adrenal and testis, both cytochrome P450 (CYP) and 3 - hydroxysteroid dehydrogenase (3β-HSD) enzyme involved in steroid biosynthesis and produces more potent steroid progesterone, 17 α hydroxyprogesteron and androstenedione (Payne and Hales, 2004). All enzymes involved in steroidogenesis are regulated by several endocrine and paracrine factors. 3β-HSD is regulated by LH receptor hence activation of LH receptor is necessary for initiation of actual steroidogenesis (Rahman and Rao, 2008). In another hands, LH secretion from anterior pituitary is necessary for stimulation of enzymes involved in steroid production therefore LH receptor plays a regulatory control over 3β-HSD expressions. In testicular tissue, LH receptor controls the proliferation and maturation of Leydig cells that is essential for testosterone production (Dufau, 1998). LHR interact with luteinizing hormone (LH) to further precede proliferation and maturation of germ cells and Leydig cells (Roess *et al.,* 2000) in adults as well neonatal.

Testis, site of steroidogenesis, is known for expressing many CYP enzymes such as CYP11A1, CYP17A1 and CYP19, in which CYP19 also known as

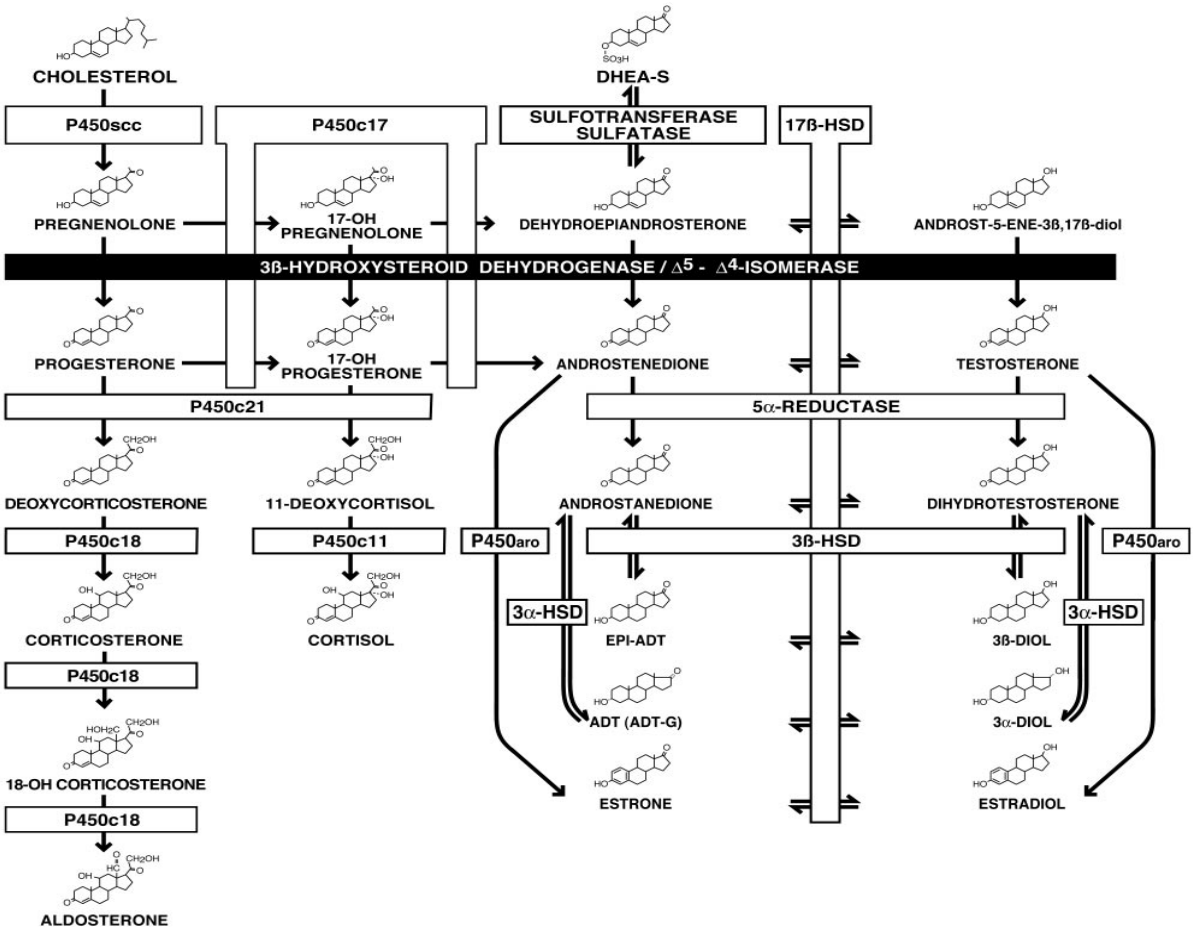


Fig. 2. Mammalian steroidogenesis pathways.

(Adopted from Simard *et al.*, 2005)

aromatase, synthesize estrogens from aromatization of testosterone (Simpson *et al.,* 1994). Notably, conversion of estrogen from androgen, which is encoded by CYP19 gene, one of the sensitive step in steroidogenesis, where elevated level of synthesize estrogen can be most documented sign of endocrine disruption in male reproductive physiology. However, some amount of estrogen in male is necessary for development and maturation of spermatozoa and low level of estrogen may lead to hypogonadism (Robertson *et al.,* 1999). Up regulation or down regulation of cytochrome P450 aromatase by any means may lead to imbalance of poor gonadal function, which is crucial for reproductive functioning as well as fertility (Li *et al.,* 2001).

1.9. Testicular function and endocrine regulation

Gonads are one of endocrine gland located in animal body responsible for reproductive development in both male and female. Testis and ovary, located in down abdomen part of body produces haploid gametes in the form of sperm and ova respectively. Development and functioning of gonad is directly controlled by hypothalamic pituitary gonadal axis (**Fig. 3**) which is down regulated or upregulated by means of their specific G protein-coupled receptors (GPCRs) like GnRH, LH and FSH receptors (Weinbauer and Nieschlarg, 1995). GnRH from hypothalamus monitors the adenohypophysis of pituitary portal system for synthesis and secretion of gonadotrophins (Gn), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Furthermore, LH and FSH receptors in testicular tissue stimulate Leydig cells

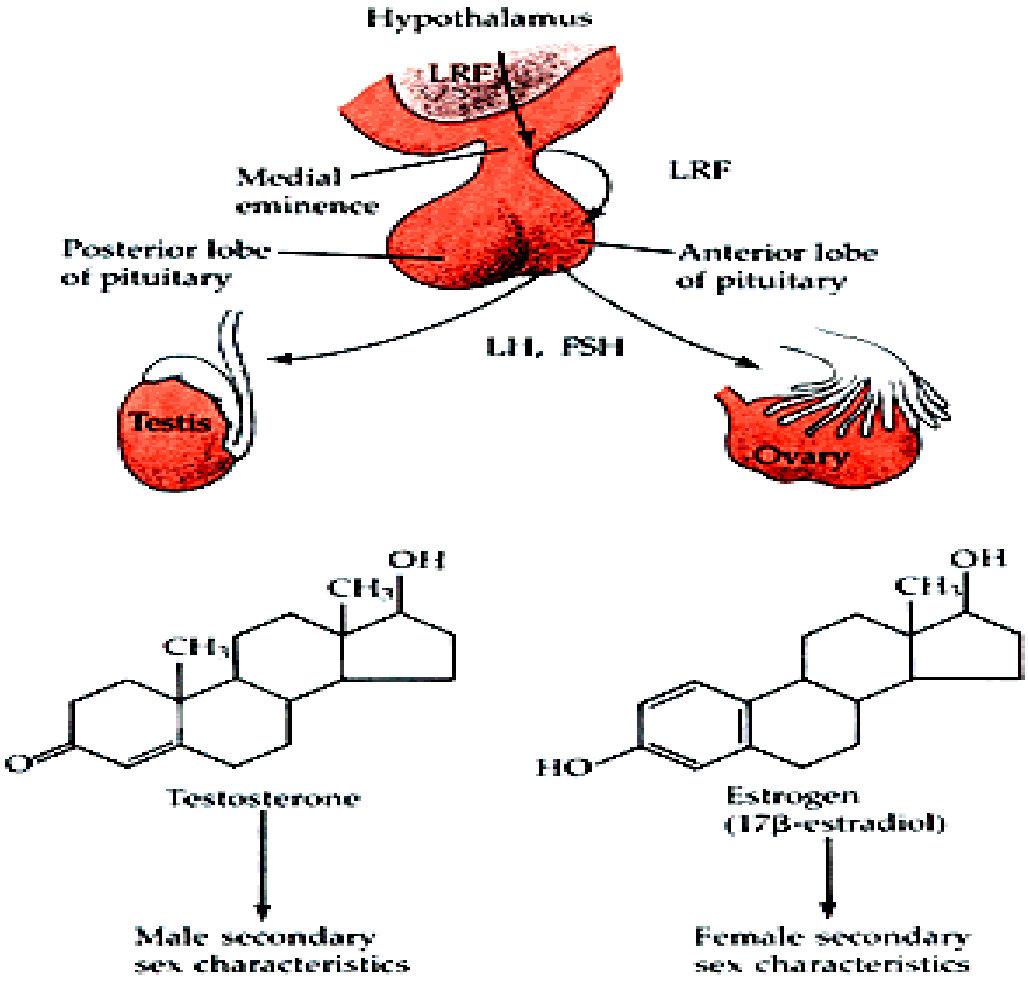


Fig. 3. Hypothalamic-pituitary-gonadal axis in mammals.

(Adopted from Gilbert, 2010)

and Sertoli cells to promote steroidogenesis and proliferation of spermatogonia to produce spermatozoa.

Further, in male, testis is targeted organ for above mentioned G protein-coupled receptor (GnRHRs) bind with their specific receptors. Activated receptors present in testis and ovary initiate the production of gametes and sex hormone.

Seminiferous tubule present in testis of male mice is consisting of different stages of germ cells such as meiotic spermatogonia, mitotic spermatocytes and spermatids (**Fig. 4**). Sertoli cells are randomly situated on basal lamina of seminiferous tubules and between the spermatogonia mother cells, well known for FSH receptor; provide necessary environment and nutrient to developing spermatozoa and other cells (Ahmad *et al.,* 1973). Besides that, Sertoli cells are also involved in proliferation of germ cells (Akingbemi, 2005; Carreau and Hess, 2010). Leydig cell are well categorized as steroidogenic cell in testis, located in interstitial space of seminiferous tubules, surrounded by various lymphocytes, macrophages, blood and lymphatic vessels, responsible for testosterone biosynthesis (Payne and Youngblood, 1995). Cholesterol is raw material for biosynthesis of testosterone in Leydig cells. Serum testosterone is reported as lesser than that of testicular testosterone due to greater requirement for its high metabolic rate. Adequate amount of testosterone is required for proliferation and maturation of germ cell as well spermatozoa (Akingbemi, 2005). Spermatogenesis is trigger in presence of steroidogenesis product testosterone, produces spermatozoa in lumen of seminiferous tubules, nourished by Sertoli cells and further moved and stored in caudal epididymis. LH receptors are

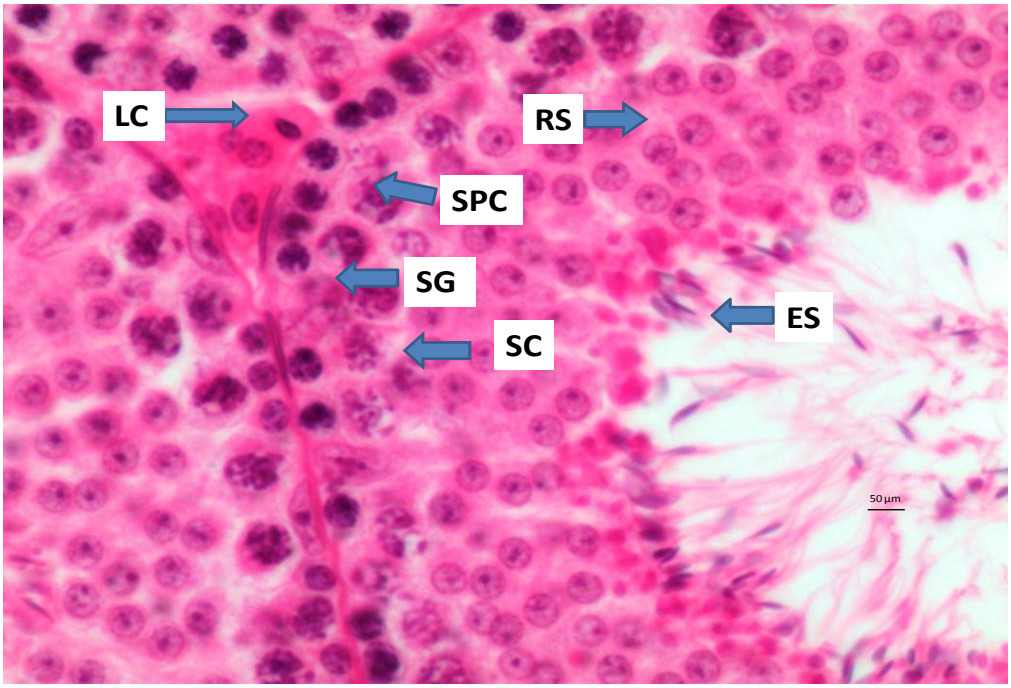


Fig. 4. A detailed view of spermatogenesis in normal mice testis.

(**LC**- Leydigs cell, **SC**- Sertoli cell, **SG**- Spermatogonia, **SPC**- Spermatocytes, **RS**- Round spermatids and **ES**- Elongated spermatids)

found on plasma membrane of Leydig cells, binds and accelerate the process of steroidogenesis. Leydig cell shows various active receptors on its membrane for testosterone production such as StAR, 3β-HSD, LH receptor and aromatase. Furthermore, in response to highly affinity FSH and LH receptors, testis and ovary produces sex hormone androgen estrogen and progesterone. Both FSH and LH are required for maintenance of homeostasis between gametogenesis and steroidogenesis (Gill-Sharma, 2001; Weinbauer and Nieschlarg, 1995). Thus, the peripheral circulation of sex hormone is strongly affected by both anterior parts of pituitary as well as hypothalamus with their specific feedback mechanism (Conn *et al.*, 1987). So, all kind of cells present inner and outer spaces of seminiferous tubules support each other for spermatogenesis and steroidogenesis, tightly regulated by hypothalamic-pituitary-gonadal (HPG) axis, necessary for healthy fertility (Donnell, *et al.*, 2001).

Not only testosterone, but also estrogen shows active response to HPG-axis for down regulation of LH and FSH receptor (Carreau and Hess, 2010). Furthermore, one of the most important roles of pituitary gonadotropins in steroidogenic pathway is to monitor the expression and production of testosterone and estradiol as it regulate the expression of cytochrome P450 aromatase (CYP19). Once, testosterone is enough produced to maintain normal homeostasis of spermatogenesis, the HPG-axis promoted a negative feedback response for stop adenohypophysial secreation of LH and FSH (Donnell *et al.*, 2001; Akingbemi, 2005). Androgen and estrogen receptor present in testis are eventually found in mitotic germ cells and non-mitotic Leydig cells and Sertoli cells involved in testicular function (Carreau and Hess, 2010).

Estrogen receptor shows a crucial role in male reproductive process as it regulates the maturation of spermatozoa. Lack of estrogen in transgenic rodents was shown arrested spermatogenesis and increased intensity of testicular necrotic cells. Interestingly, well known fact with increased level of estrogen is also associated with testicular dysfunction such as vacuolization and mitotic germ cell arrest.

Additionally, any alteration in either expression of gene or production of hormone leads to suppress gonadal physiology. A number of endocrine suppressive agents are known to interfere in the gonadal function. However, the mechanism behind the some steroid biosynthesis blockers, that known for suppressing the gonads function efficacy by decreasing production of sex steroid is still a matter of debate. However, testicular steroidogenesis may involve several pathway and mechanism causing the alteration of sex hormone biosynthesis via affecting the hypothalamic pituitary gonadal axis. Spermatogenesis is very susceptible with low level of serum testosterone, one of the major causes of decreased fertility (Mohan *et al.,* 2011). Spermatogenesis and steroidogenesis show their direct link with hypothalamic pituitary gonadal axis for down regulation and up regulation of peripheral hormone secretion. But, however, these conditions may differentially affect as in case of steroidogenic enzymes which are very susceptible with endocrine disruptor still remains unclear and unknown.

1.10. Side effects of therapeutic exogenous steroid on reproductive function

There are a lot of evidences to proven that exogenous steroid like diosgenin may be beneficial against menopausal and andropausal condition (Tada *et al.,* 2009). Ageing is a complication of adverse metabolic and physiological changes in somatic and gonadal tissue due to imbalance in production of free radicals species that produces harmful effect on cardiovascular health, bone strength, memory, reproductive function, inflammation in skin and joints etc (Sanchez-Rodriguez *et al.,* 2007). Moreover, there is also evidence that lack of estrogen may alter the metabolism of sugar and lipid. Hormone replacement therapy (HRT) is clinical application of synthetic steroid in old age people to improve their serum sex hormone deficiency (Most *et al.,* 1995). However, negative impact with estradiol supplementation is also reported due to increased intensity of cancer in reproductive parts (uterine and cervical cancer). Not only risk of cancer, it also interferes in behavioral changes such as irritability, depression and mood swing affecting the daily routine life. According to Nambu and Kumamoto, (1995), exposure of adriamycin (ADR) drug widely used as suppression of tumor to others parts of body, causes testicular atrophy and poor spermatogenesis. Furthermore, administration of 17β-estradiol for one week leading into disrupted function of testis. Moreover, empty lumen and delaminating germ cells towards lumen of tubule were reported (Gill-Sharma *et al.,* 2001). Findings of Takahashi and Oishi, (2003) revealed that there was oxidative stress mediated cell death trigger by ROS in Sertoli cells as well in Leydig cells of testis due to estrogenic effect of drug. Many other synthetic anabolic steroids used for various therapeutic purpose has been reported as anti-androgenic

drug (Nagata *et al.,* 1995) due to their inhibitory effect on spermatogenesis and steroidogenesis leading into sterility in male.

Besides gonads, adrenal gland is second most targets for alteration in cortiosteroidogenesis (Mann, 1996; Rossol *et al.,* 2001). Previous report suggested that use of a synthetic androstane, cyanotrimethyladrostenolone causes suppression of adrenal steroid hormone (McCarthy *et al.,* 1996). Another research report revealed that ketoconazole showed an inhibitory effect on cytochrome P450 thus adversely affected the adreno-steroidogenesis (Loose *et al.,* 1983). Environmental endocrine disruptor has been shown as estrogen like effect, impairs the cortisol in fish from a heavy metal contaminated water source (Hontela *et al.,* 1992) may be due to oxidative stress mediated tissue toxicity (Hornsby, 1989).

1.11. Impact of drug metabolism and related oxidative stress in testis

It is well known that drugs are frequently used for long periods of time, may interfered individual reproductive physiology because of highly sensible nature of gonads (Nambu and Kumamoto, 1995). Diosgenin is widely and frequently used as therapeutic agents for long periods of time to cure various kinds of disorder. From above aspect, there is urgent need to access its effect on gonadal function due to its steroid nature. Study by Aitken and Shaun, (2008) reported exogenous steroid interferes in intra-testicular endogenous testosterone production in testicular tissue, and raises massive production of destructive ROS. Research by Forlenza and Miller,

(2006), explained abnormal increased oxidative stress in germinal epithelia may produce abnormal spermatozoa due to elevated level of testicular MDA content (**Fig. 5**). Gonadotoxicity is also major concern with increased lipid peroxidation product as increased in concentration of free radical species adversely affect the rate of apoptosis (Agarwal *et al.*, 2008).

In earlier studies, it is well reported therapeutic agent metabolism is involved in reproductive toxicity (Orisakwe *et al.,* 2003; Obianime *et al.,* 2011) because of the increased production of ROS. There is direct link between increased high ROS production and damaging DNA, RNA, protein content, lipid level and several enzyme functions (Rosenblum *et al.,* 1989). Similar report has been observed from Doreswamy *et al.* (2004). According to him, protein biosynthesis is susceptible to elevated MDA concentration due to adduct formation with nucleic acid.By product of drug and its metabolites are known to interfere in nucleic acids function thus exert a damaging effect on DNA structure (Bhattacharyya *et al.,* 2014; Sinha *et al.,* 2013; Liu *et al.,* 2002).

Natural cellular antioxidant defense system (GSH, SOD, Catalase and GST) detoxify the end product of peroxidation and protect cells from ROS. Moreover, any imbalance in MDA: antioxidant ratio can trigger oxidative stress mediated cell death and can complicate the process of normal physiology (Deavall *et al.,* 2012). In addition to that, impaired ROS homeostasis in testis may increasesthe Bax/Bcl-2 ratio and activation of caspase-3leading into programmed cell death (Mishra and Shaha, 2005; Elmore, 2007). Moreover, abnormal activation of caspase-3 within cell

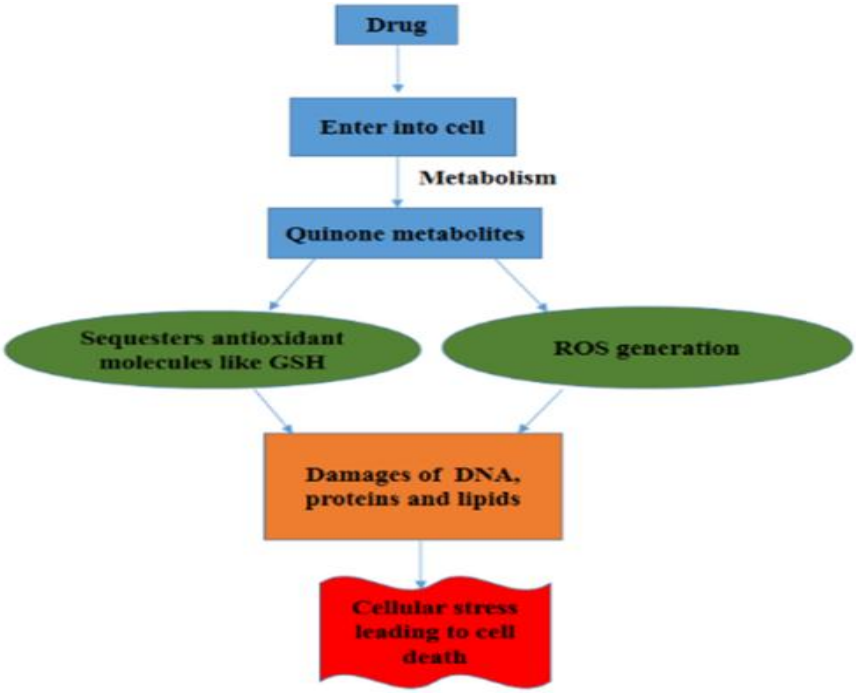


Fig. 5. Hypothetical mechanism of drug induced cellular stress oxidative stress. (Adopted from Banerjee *et al.*, 2016)

may affect the process of sperm production as excessive death of germ cell affect fertility output.

Many environmental factors (excessive heat and radiation) and certain chemicals (pesticides or drugs) may increases the process of germ cell apoptosis in testicular tissue leading into degeneration and finally cell death (McIlwain *et al.,* 2013) (**Fig. 6**). In testis, oxidative phosphorylation is required to provide continuous energy supply for spermatogenesis as well steroidogenesis. However, increased apoptosis in seminiferous tubule may suppress the testicular activity (Behrman and Aten, 1991). Furthermore, ROS may indirectly decrease the protein expression of key steroidogenic enzymes (StAR, 3β-HSD and aromatase) and receptors. In addition to that, report of Li *et al.* (2009) demonstrated a significant inhibition of steroidogenic enzymes function after BPA (widely used in food industries for packaging of food and juices) exposure leading into altered spermatogenesis.

1.12. Diosgenin absorption, metabolism stability and disposition

Absorption and metabolism of any drug is dependent on its structural constituents and nature of its fluid medium (gastric and intestinal fluid). Besides that, Bioavailability or degradation of drug is also dependent on incubation period of fluid medium. Among the well-known and popular method of estimation of drug absorption rate, the most common method is bidirectional transport of a drug across Caco – 2 cells (Artursson *et al.,* 2001). One another mechanism for investigating

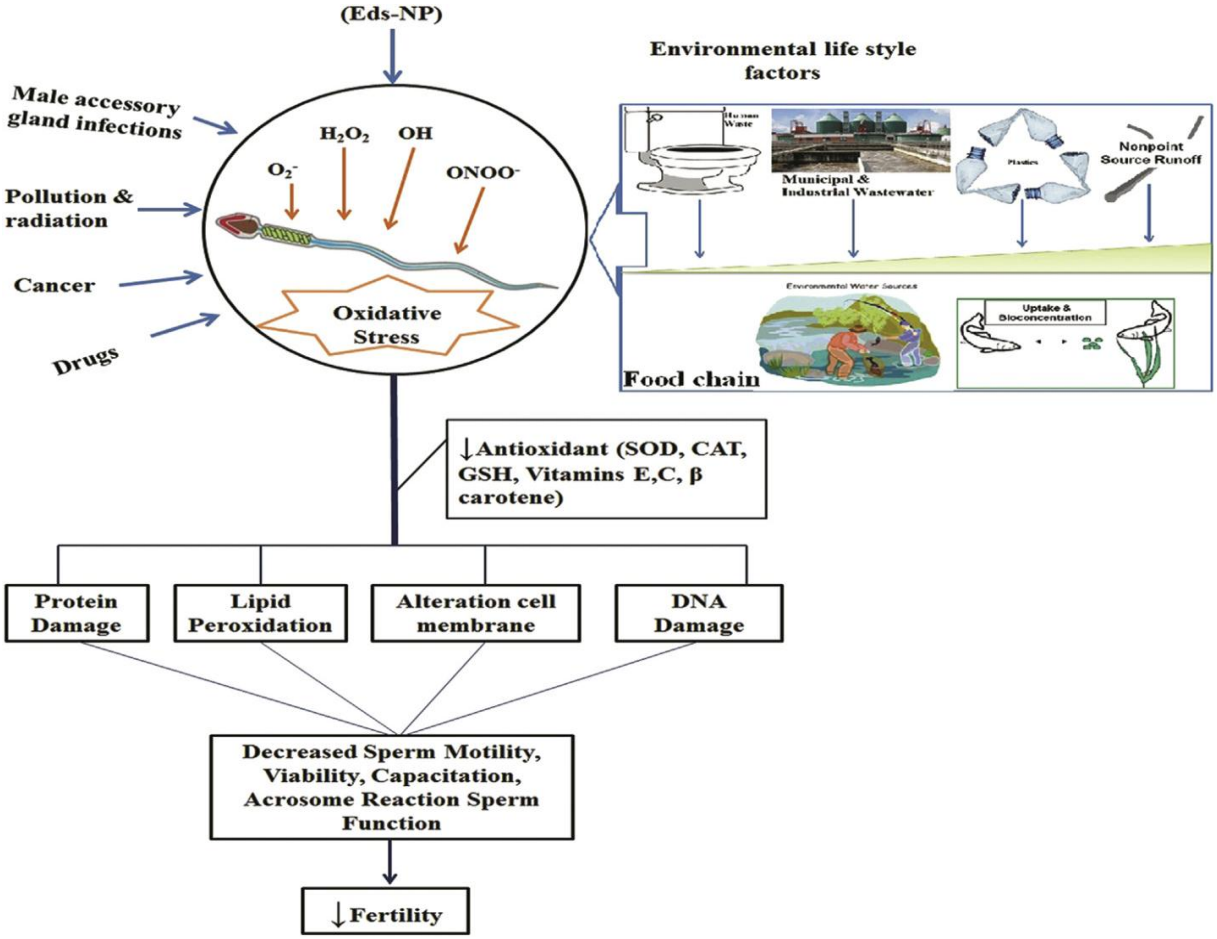


Fig. 6. Mechanism action of increased production of ROS and its effect on spermatogenesis (Lamirande *et al.*, 1995).

drug bioavailability is incubating particular drug with microsomal particle (human liver), its half-life potential and disposition (Chao *et al.,* 2010). There are many drug metabolizing enzyme, commonly known as cytochrome P450 enzyme such as 1A2, 2C19, 3A4 and 2D6 (Manda *et al.,* 2013). The amount of drug loss is well quantified with incubation of drug in biological fluid system. Moreover, Guidance of FDA (Food and Drug Administration) and Center for Drug Evaluation and Research (2000) suggested that a particular drug shows more than 5 % degradation, have less stability issues. Previous reports of Cayen and Dvornik (1979) explained about diosgenin metabolism and its elimination. According to them, diosgenin is poorly absorbed and easily eliminated via bile product. Accordance with their finding, one another research by Roman *et al.* (1995) has reported diosgenin increases biliary cholesterol and decreased biliary phospholipids via uncoupling biliary lipid and bile salt secretion. Most recently, the aglycon diosgenin and another glycoside form, dioscin, have been studied for its bioavailability, metabolism stability and disposition by Manda *et al.* (2013). Accordance of their research, they concluded that both diosgenin and its glycoside form dioscin, shows moderate permeability for intestinal mucosa across Caco-2 monolayers. The amount of diosgenin was present after incubation period suggested slow absorption rate across the intestinal mucosa when compared to its glycoside form, dioscin. Manda *et al.* (2013) also reported metabolic stability of diosgenin. The HLM (human liver microsome) and S9 fraction with a half - life of 11.3 minute, suggested that diosgenin may interfere some of cytochrome P450 enzyme by altering their activity especially at high dosage.

REVIEW OF LITERATURE

2.1. Biological role of *Dioscorea*, *Trigonella* and *Costus* species

Customary prescriptions have exhibited a splendid future in finding a new medication and to comprehend the significance of conventional herbs. A few herbal plants have been utilized as dietary supplements in the treatment of various illnesses without legitimate learning of their capacity properties and hypersensitivity. The fact encouraged to use herbal product is synthetic drug are known to cause harmful effect to reproductive function (Aarab *et al.*, 2004). However, Herbal plants have been used to treat many conditions of diseases and many times misconception of being fit and healthy, but have serious side effects which lead to deleterious effect for several systems in the body. Enthusiasm for plant-derived phyto-estrogens or different substances that influence the reproductive framework has as of late been expanded by the acknowledgment that hormone substitution treatment is neither safe nor successful.

Fenugreek is a traditional herb in India and many other countries. Fenugreek plant parts such as seeds and leaves have been utilized for sustenance also as traditional prescription. The ripe seed of fenugreek is saturated with many antioxidant compounds with medicinal property. It has several complementary medicinal uses such as lactating agent for milk production after delivery, pain reducer, anti-wormicide, sugar and fat lowering property (Srinivasan, 2006). The nature of fenugreek seed is mild laxative and diuretic. It has been used in treatment of leprosy, hemorrhoids, as mouth spray, digestive complication and in several kinds of infection. Besides these, several other studies have reported fenugreek as anti-

inflammatory and antipyretic (Ahmadiani *et al.,* 2001), anti-diabetic (Jayadev *et al.,* 2001; Thakran *et al.,* 2004; Kumar *et al.,* 2005), hypocholesterolemic (Abdel-Barry, 1997), antifungal (Haouala *et al.,* 2008), anti-bacterial (Sudar and Kirti, 2008) and immune modulatory (Ramesh *et al.,* 2002; Bin-Hafeez *et al.,* 2003). The seeds contain its major active steroidal saponin diosgenin (Taylor *et al.,* 2002).

Diosgenin was first time reported in yam species (*D. tokoro*) and later prescribed as precursor for synthetic steroid production (Fujii and Matsukawa, 1936; Marker, 1940). *Dioscorea* has been locally used to as pain reliever, gastric purpose, to increase sex desire and potential. The rhizome of *Dioscorea* consist of various kind of biologically compound in which a potent saponin, diosgenin is major active compound. Previous coworkers reported use of wild yam as dietary supplement can reduce colic pain (Gunn, 1859), rheumatoid arthritis (Crellin and Philpott, 1990), menstrual pain (Cook, 1869), uterine cramps (Moerman, 1998), pseudo labor pain (Fyfe, 1909), nausea (Warren, 1859), gut inflammation (Howell, 2006) and excess gastric flatulence (Cook, 1869). Besides that, it has been also used as cholecystitis, ovarian neuralgia, bowel disorder, expectorant, salpingitis, diverticulitis, neuralgia, body cramps, claudication and to reduce symptoms of aging. Furthermore, another therapeutic use of *D. villosa* is to improve reproductive function (Ellingwood, 1919). Another report claimed that use of yam extract can cure disorder of nervous system and brain (Paine, 1874). However, continuous use should be avoided due to severe side effects of yam (Ellingwood, 1919). Recent work has revealed that yam species such as *D. bulbifera* may be useful to treat hyperglycemia and hyperlipidemia due to

its suppressive effect against increased blood sugar and lipid level (Ghosh *et al.,* 2014; Son *et al.,* 2007).

Another source of diosgenin has been reported in rhizome of *C. speciosus* (Sarin *et al.,* 1974). Traditional uses of rhizome extract has reported as fever reducer, effective in dysentery (Srivastava, *et al.,* 2011), joint pain reliever (Ariharan *et al.,* 2012) and wound healing property. Other folk medicinal uses are as anti-cough remedy, antidote for venom, softening of sever hard stool, anti-inflammatory in lung diseases (Hasan and Qari, 2010) anti-nausea, to clean intestinal worms (Srivastava *et al.,* 2010) and as contraceptive purpose (Choudhury *et al.,* 2012). The rhizomes extract are reported as anti-fertility, to increase sexual behavior and uterine muscles relaxant (Choudhury *et al.,* 2012). Moreover, *C. speciosus* has been shown inhibitory effect on hyperglycemia and hyperlipidemia in experimentally induced diabetic rat model (Bavarva and Narasimhacharya 2008; Rajesh *et al.,* 2009). In another study, Jha et al. (2010) revealed that rhizome extract of *C. speciosus* shows antioxidant and cytotoxic property. Moreover, anti-microbial property of *C. speciosus* has been investigated by Duraipandiyan et al. (2012). Stress reducer property of *C. speciosus* has been studied by Verma et al. (2009). According to him, alcoholic rhizome extract of *C. speciosus* reduces the brain stress parameters in rat. Report of Dubey *et al.* (2010) has revealed diuretic property of rhizome. Moreover, recently, antifertility property has been reported in rhizome extract of *Costus* (Choudhury *et al.,* 2012; Kagbo and Obinna, 2017, 2018).

2.2. Reproductive toxicity of saponin diosgenin

Although, saponin is customarily viewed as sheltered and much endured, but there is reproductive toxic impacts that have been related with fertility on account of quality of steroid saponin (diosgenin). Diosgenin, an imperative source for crude material for the industrial creation of steroid drugs e.g. testosterone, progesterone and cortisone (Djerassi *et al.,* 1952). Some adverse role of saponin (diosgenin) has been documented for male reproductive function as well female reproductive function.

2.2.1. Male antifertility activity

Diosgenin is the most potent bioactive compounds abundantly found in some leguminous plants widely used as alternate source of estrogen by menopausal women to avoid severe side effect of synthetic steroid (Aarab *et al.,* 2004). In another hands, one of the significant activities of antifertility herb is their impact on sperm physiology that influences capacitation of sperm responsible for fertilization. Steroidal sapogenins (diosgenin) present in fenugreek are known to exhibit estrogenic and androgenic activities and causing impaired reproductive function and teratogenicity (Al-Yahya, 2013, Dande *et al.,* 2012; Kassem *et al.,* 2006). As of late, it is observed that spermatozoa bring down results in sperm motility and count and found to prompt critical changes in the chromosomal abnormalities in fenugreek treated animals (Al-Yahya, 2013). In addition to that, another report on crude non-polar steroidal portion of fenugreek extract demonstrated complete infertility, huge

decreases in reproductive organ weights, sperm counts and motility (Kamal *et al.*, 1993).

Diosgenin (steroid) shows structural similarity with estrogen. It may interfere in aromatization of testosterone into estradiol (E2) which leads to expanded aromatase action, bringing an expanded level of estradiol. Previous results demonstrated that fenugreek seed extract fundamentally diminished testosterone level in rabbits. Furthermore, fenugreek exposure in mice demonstrates expanded plasma estradiol and decrease of testosterone, which are known to influence the fertility. *Dioscorea* contains saponin (diosgenin), known to cause reproductive impairments (Sato *et al.*, 2007). Work reported by Mohan *et al.* (2011), revealed that there is dose dependent effect of *Dioscorea* on reproductive system. According to him, ethanol extract of *D. esculenta* significantly inhibited all sperm parameters along with marked decreased serum testosterone, which is crucial factor for male fertility. However, yam tuber powder has been traditionally used to increase sperm quality and quantity (Neelima *et al.*, 2011; Radha *et al.*, 2013) in case of reproductive disorder of male. In another case, there was change of the male reproductive hormones together with diminished in mass, motility and typical morphology of the sperm caused by *T. foenum-graecum* seeds extract (Khare *et al.*, 1983). It indicated comprehensive impacts of fenugreek on male reproduction which included oxidative stress in the gonads and dysfunction of reproductive hormone system may be connected with the generation of oxygen radicals.

Hypogonadism is a consequence of dysfunction of gonads. Testis is site of steroidogenesis, viewed as increasingly influenced pressure due to its high metabolic

necessities. It is related with lopsidedness of reproductive hormone as low testis weight resulted in poor spermatogenesis (Morales and Heaton, 2001). Diets containing 30% fenugreek seedsin male diminished testis weight and also circulating testosterone (Kassem *et al.,* 2006). Similarly research was done by Ibrahim and El-Tawill, (2010). According to them, fenugreek seeds powder (200 mg/rodent every day) for 30 days in male rodents have demonstrated all the more declining gonads (testicles) alongside huge decline in serum LH and testosterone hormones, indicated testicular toxicity (Ibrahim and El-Tawill, 2010). These findings showed a harmful effect of fenugreek seeds on seminiferous tubules leading into reduced testis weight. These imbalances of reproductive hormone caused by under active testis further weakened the ability of sperm formation, maturation and transport.

Sperm morphology is one of sensitive parameter of sperm quality analysis. Compounds such as steroid saponin can modify normal sperm morphology. A few studies on male rodents have appeared that fluctuation in reproductive hormone is possibly significant cause for decline sperm quality. Recently, treatment of male mice with ethanol extract of fenugreek (100 mg/kg/day body weight) for 90 days has been shown to increases sperm head and tail abnormalities (swollen acrosomes, amorphous, microcephaly, megacephaly, rotated head and flat head) and an increase in the amount of abnormal sperms related with DNA damage (Al-Ashban *et al.,* 2010; Al-Yahya, 2013).These results suggested that the DNA damage and sperm morphology reflected hereditary damage in the male germ cells may be because of high level accumulation of free radicals (Al-Ashban *et al.,* 2010; Kumar *et al.,* 2002).

Spermatogenesis and steroidogenesis are two principle function of mammalian testis. An imbalance in reproductive hormone system bound to influence spermatogenic events. The testis is considered to be more associated with environmental stress causing impaired spermatogenesis. It is well reported that decline in serum testosterone level is demonstrative of interrupted spermatogenesis. A Past study reported huge modifications in testicular histopathology of seminiferous tubules in male rodents supplemented with fenugreek seed (100 mg/kg b.w. / day) for 60 days (Mohammed *et al.,* 2013). Kassem *et al.* (2006) has reported that fenugreek supplementation for 3 month in male rabbit's causes testicular toxicity (degeneration of seminiferous tubules) accompanied with low testicular weight (Dande *et al.,* 2012). In another report, testicular toxicity followed with degenerating germinal epithelia along with severs necrotic changes and prominent interruption of the interstitial stoma has been observed (Ibrahim *et al.,* 2013). Furthermore, more comprehensive research done byAl- Ashban *et al.* (2010) and Al-Yahya, (2013) reported morphological abnormality of sperm due to impaired spermatogenesis. It is well known that histological detriment in testes bound to affect spermatogenesis. The hazardous effects of fenugreek seed administration on male fertility to be crucial in spermatogenesis process as all sperm parameters such as sperm motility, viability, concentration and morphology were also affected with impaired spermatogenesis. There are few laboratory studies on effect of *Costus*rhizome extract on male reproductive system (Yakubu *et al.,* 2008; Ebeye *et al.,* 2015). Kagbo and Obinna, (2017) has revealed that stem extract of *C. lucanuscianus* significantly suppress the spermatogenesis and induces the morphological alteration of sperm in male.

A few examinations have demonstrated fenugreek to be a strong antifertility herb in a few imminent and animal model studies. Fenugreek treated male mice might be showed lower acrosin activity, require for prosperous fertilization (Zalata *et al.*, 2004). Oxidative stress might be one of the fundamental mechanisms through which exposure to fenugreek can adversely influence sperm functions and cause fertility issues. Supplementation of fenugreek seed for a time of 90 days at doses 305 and 610 mg/kg/b.w. in mice diminished fertility with noteworthy lessening of immotile sperms related with DNA damage (Al-Yahya, 2013). These findings were similar report of Yakubu *et al.* (2008) and Kagbo and Obinna, (2017). They investigated that use of rhizome extract of *Costus* may reduce the fertility potential. However, a detailed investigation is required to find out the exact androgenic and anabolic action and related molecular mechanism of reproductive alterations.

2.2.2. Female antifertility activity

Female mice normally shows a range of 5–6 day reproductive cycles in which estrus phase is known as fertile phase. The duration and length of these phase types determined by ovarian activity. There is lot of evidences that proven exogenous steroid significantly altered the reproductive cycle and function in female individual. Attributions of ovarian weight and serum reproductive hormone in female are considered the most important characteristics for female fertility evaluation, since ovary is responsible for folliculogenesis and steroidogenesis. Several studies in female with fenugreek treatment revealed decreased in uterine and ovarian weights

together with significant reduction in female hormone levels (Ibrahim, *et al.,* 2010; Sharma *et al.,* 2005) and markedly increased in uterine diameter in immature ovariectomized rats (Dande *et al.,* 2012). Most recently, report of Kagbo and Obinna, (2018) revealed that rhizome of *Costus* which is used as alternative source of estrogendue to presence of steroid saponin, suppress the serum estrogen level and interrupted the phases of reproductive cycle. Previously, Khare *et al.* (1983) reported saponin present in fenugreek exhibit mild antifertility herb in female rats. In another hands, yam species are known for alternative source of estrogen in case of menopausal disorder (Wu *et al.,* 2005) has been reported for its adverse effect on normal reproductive function. Moreover, remarkably there was some proliferative changes associated with endometrial glands with hyper plastic changes, may interfere implantation activity. Recently, Ibrahim and El-Tawill, (2010) reported remarkable degeneration of some ovarian follicles after 30 days treatment of fenugreek in female mice. These observations are comparable with the studies made by Ahirwar *et al.* (2010) and Modaresi *et al.* (2012). According to them, there was stopped folliculogenesis trend and ovary tissue destruction in female Balb/C mice supplemented with fenugreek seed is might be due to estrogenic activity that may affect ovarian folliculogenesis (Sreeja, 2010). Report from Yakubu*e et al.* (2008) and Ebeye *et al.* (2015) has suggested *Costus* as antifertility plant due to inhibitory effect on female reproductive system.On the other hand, Morgan, (2011) has revealed that *D. villosa* significantly improve the side effect of hormonal imbalance after ovariectomy (Tucci and Benghuzzi, 2003). The extract of *Dioscorea* rhizome has known for estrogenic effect due to its potential to stimulate the uterine tissue to increase in mass (Das *et al.,* 2014).

A tremendous group of preclinical exploratory proof proposes that saponin diosgenin present in fenugreek seed powder to rodents during the initial ten days of gestation leading into abortificient effect (Elbetieha *et al.,* 1996). Report from Choudhury *et al.* (2012) revealed that it has been traditionally used as contraceptive purpose as well to improve reproductive complications (Bala *et al.,* 2014). Another report claimed that (Das *et al.,* 2014) *D.bulbifera* plant has been traditionally used by female for contraceptive as well abortificient activity. *D.bulbifera* plant paste is traditionally used by female for contraceptive as well abortificient activity. In another reports, ethanol extract of fenugreek administration in female rat and mouse exert anti-implantation and abortificient activity (Ahirwar *et al.,* 2010). This study correlates with several works such as that of Dande and Patil, (2012) who reported that fenugreek affect implantation activity, leading to re-absorption of fetus (abortificient activity). However, accordance with the finding of these and because of the complexities in the behind reproductive procedures included, a few new investigations should be led. Numerous researches have been done to access role of estrogenic compound present in various herbs during gestational development. Most recently, Kagbo and Obinna, (2018) explained effect of methanol leaf extract of *Costus* on gestational development of fetus in pregnant rats and reported that there were no obvious changes in offspring. Several clinical studies on teratogenic effect of fenugreek addressing fetus deformities (Sethi *et al.,* 1990), increased mortality in rats (Sabzevari *et al.,* 2002; Araee *et al.,* 2009), marked decreases in fetus and placental weights (Dande *et al.,* 2012), limb bone disorder (Skalli *et al.,* 2006), malformations such as arrangement of congenital fissure and a knock on head in infants alongside modified neurobehavioral issue (Khalki *et al.,* 2010, 2012),

increased in percentage of dead embryos. Moreover, in human case, maternal consumption of fenugreek seeds causes several distinct congenital deformities such as hydrocephalus; anencephaly, cleft palate and spinal bifida (Rguibi *et al.*, 2006). There has been report that infants show a typical smell after maternal consumption of fenugreek just before delivery (Korman *et al.*, 2001; Sabzevari *et al.*, 2002). It is suggested that estrogenic compound (diosgenin) may transported though placental barrier, affecting growth and physiology of developing fetus (Khalki *et al.*, 2012).The exact mechanism of embryo toxicity of fenugreek steroid has not been well specified. However, animal studies suggest that this steroidal saponin might interfere in maternal physiology, affecting fetus development. Keeping all these reports in view, fact encouraged us to take up diosgenin for detailed exploration specifically on the reproductive function in male reproductive system.

2.3. Phytosteroid, estrogenic activity and its effect on male reproduction

Herbal plants are widely available, cheap and abundantly found in nature, used and consumed as folk medicine in worldwide. Herbal medication is not as safe as we think. There is various kind of side effect with excess consumption due to sensitive nature of reproductive system. Food rich in phytosteroid is very effective in case of menopausal problem in woman as reported by Institute for Environment and Health (1997). Chinese women consume more soya product contain phytosteroid has less postmenopausal problem comparison to European women. Phytosteroid contains compound that exert structural similarity with cholesterol, progesterone and

estradiol. Hormone replacement therapy is clinical application of estradiol in old age women to improve serum estrogen deficiency. The more likely cause of this disorder in women is menopause which is consisting of several metabolic and physiological changes in somatic and gonadal tissue. Menopause consist of a series of changes in woman body such as osteoporosis, vaginal dryness, obesity, memory loss, cardiovascular disorder, skin problem, elevated level of cholesterol and triglycerides due to lack of estradiol. Estrogen, more likely known as female sex hormone, control and monitors the sexual cycle and other important function in body. Beside its primary and secondary sexual characteristics it shows a variety of pharmacological function such as in case of bone loss, to improve brain memory power, to increases sex drive, for healthy vascular function . Several clinical and animal studies remain to be discussed on estradiol supplementation as there is report on increased intensity of cancer in reproductive parts (uterine and cervical cancer). Not only risk of cancer, it also interferes in behavioral changes such as irritability, depression and mood swing affecting the daily routine life of women. Males have quite different reproductive physiology as compare to female have. It is devoid of phases of reproductive cycle. In case of male individual, there is regular series of events happening for development of male gametes called, spermatogenesis. Males do not have any menopausal condition as like female but with ageing there is decline in testosterone formation leading poor fertility potential. So, it is matter of debate whether phytosteroid like diosgenin, known for its estrogenic activity (Aradhana *et al.*,1992) can be effective in case of male ageing still leave lots of confusion. Research finding by Yu *et al.* (2011), revealed diosgenin might rescue the reproductive activity against D-galactose-induced aging male rats via up-regulated

the all sperm parameters (motility, concentration and daily sperm production). However, whether, the protective role of diosgenin is due to its strong antioxidant nature or estrogenic drug nature is still not well clear. The mechanism and related physiological changes at molecular level or at endocrine level (pituitary – gonadal axis) need to be research. Several clinical and animal studies remains to be discussed on the diosgenin effects in the male reproductive system because since in case of several disorders that misbalance testicular function, diosgenin shows numerous protective effects against testicular damaged but lacking information on role of diosgenin in spermatogenesis and steroidogenesis.

2.4. Recent studies of diosgenin: A biological and pharmacological analysis

Diosgenin, an active phyto-constituent of fenugreek (Fazil and Hardman, 1971; Petropoulos *et al.,* 2002) and many variety of yam, well known in pharmacology for raw or initial substance for manufacturing the industrial production of synthetic progesterone, testosterone, estrogen and corticoids due to its structural similarity (Rosenkranz *et al.*, 1951).The known sources of its occurrence in fenugreek, wild yams and *Costus* species was exposed by Fujii and Matsukawa, (1935). Recently, its therapeutic and pharmacological activity has received a lot of attention in various pathological conditions such as disorder in metabolism of sugar and fat and related organ damage difficulty. Besides that, it has been used as anti-proliferative drugs in case of breast cancer (He *et al.,* 2014; Bhuvanalakshmi *et al.,* 2017), gastric cancer (Mao *et al.,* 2012), lung cancer (Mohammad *et al.,* 2013) and

liver cancer (Li *et al.*, 2010) etc. The key point for its anti-proliferative property is its phenyl r moiety structure that improves their capacity for cytotoxicity of cancerous cells. Furthermore, Diosgenin, being a precursor of several synthetic steroid hormone productions, is well known option for menopausal (premenopausal and postmenopausal) disorder in aged women. In case of male aging disorder, diosgenin administration in aging rat model improves volume and density of bone as in case of osteoporosis (Ho *et al.*, 2014). Most recent, diosgenin exposure reveals the altered sexual dysfunction by D-galactose induced aging (Yu *et al.*, 2011). However, there is plenty of research on diosgenin and male reproductive function warranted to find out. Herein, some of metabolic diseases and other condition of diseases has summarized below with possible protective action of diosgenin.

2.4.1. Diosgenin and disorder of sugar metabolism

Diosgenin, being a bioactive component of various kinds of yam and fenugreek, well categorized as metabolism regulating drug in pharmacology. Since ancient time, fenugreek seed (whole or its extract) and yam extract has been used to treat hyperglycemia. Presence of its sapogenin compound, diosgenin, makes it possible due to its ameliorative role. Several glucose metabolizing enzyme such as Hexokinase and Glucose – 6 – phosphatase play a potential role in glucose uptake and hepatic gluconeogenesis respectively. In experimentally induced diabetic animal model, diosgenin shows modulating effect on sugar regulatory enzyme of kidney and liver leading into significantly improve elevated plasma glucose possibly via increasing the glycolysis rate and limiting glycogenesis (McAnuff *et al.*, 2005;

Naidu *et al.,* 2015; Saravanan *et al.,* 2014). In case of impaired blood glucose level due to insulin deficiency, diosgenin is known as very effective drug against impaired insulin formation (Kalailingam *et al.,* 2014). Hyperglycemia due to insulin resistance or degeneration of pancreatic beta cells is associated with several kind of health risk in diabetic patient and seriously affects the working capacity of other body organs. In case of diabetic related organ damage problem, diosgenin significantly protect from oxidative stress exerted due to increased free radical in coronary artery disease, myocardial and aortic injury, degenerated beta cell of pancreas, kidney failure , eye sugar cataract and elevated level of bad cholesterol, neurodisoder (Kalailingam *et al*., 2014; Pari *et al,*. 2012; Northam *et al.,* 2006). Furthermore, it has been experimentally proved by Ji *et al.* (2017), diosgenin acted as an Aldose reductase (AR) Inhibitor in galactosemic cataract animal model. It is well known that AR is stimulated by elevated blood sugar and its activation leading the sugar cataract. Another report investigated that diosgenin stimulates muscular GLUT4 signaling pathway in case of type 1 diabetes, leading to increase in DHEA formation, reduces high blood sugar level (Sato *et al.,* 2014). This effect of diosgenin is due to its structural similarity with many steroid hormones, increases muscular steroidogenesis and increases in the DHEA level, may possibly stimulate the muscular GLUT4 to improve elevated blood sugar.

2.4.2. Diosgenin and disorder of lipid metabolism

Diosgenin found in yam and fenugreek was traditionally used in Chinese and Indian to maintain plasma cholesterol and reduce obesity. As saponin, diosgenin is extensively used in pharmacology for various purpose of metabolic disorder like

disorder of lipid metabolism. Lipid metabolism is complex series of breaking down of fat into fatty acids and glycerol. Any alteration either by increased oxidative stress generate huge amount of ROS, or impairment in pathway of lipid metabolizing enzyme system, can elevate serum triglycerides and bad cholesterol (O'Malley, 1984). It is well known high plasma level of cholesterol is known cause of atherosclerosis and its related complication. Previous work reported by Cayen and Dvornik, (1979) has revealed diosgenin action on cholesterol metabolism. According to him, diosgenin interferes in cholesterol uptake and storage possibly via eliminating the cholesterol in stool and hence reduces the risk of hypercholesteromia. These finding is justified with research done by Kwon *et al.* (2003), as diosgenin significantly reduces the rate of fat absorption. Impaired lipid metabolism involved a number of limiting factor such as insulin sensitivity, kidney related problem and under working thyroid, leading a risk for cardiovascular disease. Arthrosclerosis, hypertriglyceridemia, dyslipoproteinemia and finally obesity is example of impaired lipid metabolism (Sobel, 1973). In experimental animal model, diosgenin significantly reduces bad cholesterol and increases healthy cholesterol when administered in diet Juarez-Oropeza *et al.* (1987) and Son *et al.* (2007). Recently, more comprehensive research done by Naidu *et al.* (2015) concluded that diosgenin may protect the impaired lipid profile in case of insulin resistance possibly via direct interfering in lipid metabolizing enzyme system.

2.4.3. Diosgenin and disorder of oxidative stress

It is already well known that oxidative stress is hallmark for generation of free radicals and many other ROS. However, it is a routine part of normal homeostasis between tissue growth, degeneration and regeneration. Moreover, higher production of ROS generates the risk of tissue damage and suppresses the working potential of various exocrine and endocrine glands. Several disorder of metabolic syndrome (diabetes mellitus, obesity, hyperlipidemia and hypercholestomia etc.), drug metabolism and many other environmental factors are responsible for production of free radicals seriously affect antioxidant enzyme efficacy. Now days, diosgenin is well known for its strong antioxidant effect in pharmacology industry (Al-Matubsi *et al.,* 2011), commonly used for precursor for steroidal drug (Aradhana *et al.,* 1992) and for contraceptive (Zenk, 1978). Being an active phytochemical of yam, diosgenin exerted ameliorative role against diet induced high cholesterol in rat (Son *et al.,* 2007). In case of experimentally (streptozotocin) induced diabetes, diosgenin acted as potent antioxidant and protect aorta damage from free radical species (Pari *et al.,* 2012). On other hand, diosgenin stimulates insulin production in endocrine part of pancreas (beta cells) via scavenging the ROS, hence, protect from cardiovascular risk (Kalailingam *et al.,* 2014). Additionally, diosgenin exert protective effect against hepatic oxidative stress, lipid peroxidation and molecular alterations in case of chronic renal failure rats (Manivannan *et al.,* 2013).

2.4.4. Diosgenin and disorder of cell proliferation in tumorous cells

Diosgenin has shown strong inhibitory effect against various kinds of tumorous cells and it significantly reduces the cell proliferation and their invasion towards other tissues and organs (Raju *et al.,* 2004; Malisetty *et al.,* 2005; Miyoshi *et al.,* 2011; Srinivasan *et al.,* 2009; Yan *et al.,* 2009). This inhibitory effect of diosgenin against cancerous cells is due to its cytotoxic effect. Diosgenin significantly induces apoptosis against human osteosarcoma cells and inhibited its proliferation (Corbiere *et al.,* 2003). Furthermore, diosgenin was successfully able to arrest human chronic myelogenous leukemia and significantly inhibited osteoclastogenesis, invasion, and proliferation through the down regulation of Akt, IκB kinase activation and NF-κB-regulated gene expression (Shishodia Aggarwal, 2005, 2006). Previous report of Moalic *et al.* (2001), reported inhibitory effect of diosgenin against osteosarcoma via down regulating COX – 2 functions. Besides that, diosgenin inhibited the rate of proliferation as in case of human breast adenocarcinoma cells possibly via arrest at sub-G1 phase (Chiang *et al.,* 2007). Additionally, same manner, diosgenin induces apoptosis via caspase-3 activation and arrest cell division to inhibit proliferation of hepatocellular carcinoma (HCC) (Li *et al.,* 2010; Liu *et al.,* 2005). In case of human cervical cancer, diosgenin shows its anti-proliferative and apoptotic affect in vitro (Fernández-Herrera *et al.,* 2010). Moreover, Chen *et al.* (2011) revealed diosgenin action against human prostate cancer. According to them, diosgenin, a steroidal saponin found in yam, significantly inhibited the rate of migration and invasion of cancerous cells. Recently, Bhuvanalakshmi *et al.* (2017) has reported diosgenin was able to inhibit breast

cancerous stem cells. These finding may appreciate the use of diosgenin as a potent drug against various kind of tumor proliferation and cancerous cell development.

2.4.5. Diosgenin and disorder of inflammation and immunity

Diosgenin is steroidal saponin having structural resemblance with many of steroid hormone such as progesterone, estradiol, testosterone and corticoids. Steroid used as therapeutic purpose in pharmacology industry due to its anti-inflammation action. Besides that's, its anti-inflammation property along with development of immunity has been used in various kind of immune disorder. Hence, being a steroid precursor in various pharmacological production of drug, diosgenin exert anti-inflammatory and immunomodulatory effect (Shishodia and Aggarwal, 2006; Kim *et al.*,2016; Turchan-Cholewo *et al.,* 2006; Jung *et al.*,2010). Previous report by various workers has well explained about diosgenin as inhibitor of many inflammatory factors (JNK p65/p50, p38MAPK, AP-1, NF-κB and CK2) and down regulates the tissue injury (Jung *et al.,* 2010; Gao *et al.,* 2013; He *et al.,* 2012). Anexperimental study by Wang *et al.* (2015) has suggested that diosgenin can be used as therapeutic agent to cure osteoarthritis as it able to inhibit expression of inflammatory mediators (interleukin-1β). In addition to that, Wang *et al.* (2017) has explored diosgenin acted as neuroprotector via suppressing the expression of pro-inflammatory factor M1 however, M2 markers were remain unaffected. Moreover, Turchan-Cholewo *et al.* (2006) revealed the diosgenin may act as immune booster as in case of HIV infection thus reduces the risk for dementia. In another research report by Kim *et al.* (2016)

has explained diosgenin enhanced its anti- inflammatory potential against phthalic anhydride induced skin inflammation in IL-4/Luc/CNS-1 transgenic mice. Recently, diosgenin administration has improved the formation of intestinal T helper 1-like regulatory T cells, showing the anti-allergic effect against food allergy (Huang *et al.,* 2017).

2.4.6. Diosgenin and disorder of aging

Diosgenin containing food such as yam and fenugreek have been traditionally used worldwide to secure aging related disorder. Aging is complex series of event in every living being that comes with many metabolic and physiological changes in body. Menopause and andropause is term used for aging both in female and male respectively. Menopause in female is more complex then andropause and bring sudden fluctuation in hormone homeostasis and alter glucose and lipid metabolism leading into various physiological changes. The main drawback of menopause is estrogen deficiency that significantly affects the bone loss, mental health, cardiovascular health, weight gain, joint pain and skin heath. Whereas in male, andropause is gradual series of aging leading decreased testosterone that significantly affect individual health status. Imbalance between oxidative stress and antioxidant enzyme defense mechanism, leading into excessive production of ROS cause aging related disorder. Diosgenin, present in yam due to its strong antioxidant nature may protect from aging related disorder (Chiang *et al.,* 2011). Diosgenin have potential to suppress the postmenopausal osteoporosis in ovx rat model (Higdon *et al.,* 2001).

According to Tada *et al.* (2009), diosgenin administration may reveal aging caused skin health problem. Furthermore, it is hypothesized by Ho *et al.* (2012) that chronic exposure of 10mg/kg/day diosgenin may revealed the menopausal related health risk of brain and nerve functions in menopausal rat model. Recently, in addition to previous report, Hung *et al.* (2014), has revealed diosgenin increases the frame and femur volume and decreases the porosity and frame density in D- galactose-induced aging rat model thus suggesting a good therapeutic drug for older people. Recently, diosgenin administration in menopausal rat model induces changes in adrenal gland suggesting a potential remedy for older aged people (Ajdˇzanovi´c *et al.*, 2016). These finding could be beneficial for diosgenin application in aging related clinical study.

2.4.7. Diosgenin and disorder of fertility

Diosgenin is well known in pharmacology industry as starting material for various kinds of synthetic steroid hormone and oral contraceptive due to its structural similarity with cholesterol and other steroid hormone. Fertility is health status of an adult individual and easily affected by various kind of known and unknown factors. Several kinds of environmental factor and over doses of active photochemical present in numerous foods may affect reproductive ability leading into poor fertility potential. Yam and fenugreek are used in daily food basis in worldwide; contain its active phytochemical, diosgenin. Natural diosgenin found in yam and fenugreek is being used by Chines and Indian women to treat female related sexual problem like

menopause, bleeding disorder, abdominal cramp and in delivery. First of all, diosgenin as estrogenic agent in mammalian epithelium was reported by Aradhana *et al.* (1992). According to him, diosgenin exert estrogenic effect in case of ovariectomized animal model. Fenugreek and yam contains an active phyto-constituent, altered sexual parameter in rodents is well reported previously. Furthermore, it has been reported by Medigović *et al.* (2014), that diosgenin does not affect the uterine volume and weight and hence, enable to act as estrogenic agent. Most recent, diosgenin exposure reveals the altered sexual dysfunction by D-galactose induced aging in rat. There is major doubt for diosgenin action on reproductive functions whether diosgenin has estrogenic property or anti-estrogenic property, is still remaining unclear. Moreover, its working mechanism in steroidogenic pathway will be quite similar both in male and female, leading questions and a new field of research.

2.5. The present thesis

Although, a lot of research has been conducted on novel effect of diosgenin for various diseases prospective, but still there is insufficient information on side effects of using diosgenin and diosgenin based products. Herein, we targeted to find out role of diosgenin in spermatogenesis and steroidogenesis and its effect on fertility potential in Swiss albino mice. Very interestingly, in this study we evaluated diosgenin cause reproductive alteration in normal mice. Although, due to limitations of our study, the actual molecular mechanisms by which diosgenin causes male reproductive toxicity, is remain largely unknown. However, it is well clear that

exogenous hormone (steroid) may interfere in normal male reproductive health. The possible way of diosgenin interfering in reproductive pathway is mainly at the gonadal level as well as hypothalamic-pituitary-gonadal axis. It seems diosgenin being a steroid similarity with estrogen may directly down regulate or up regulate steroidogenesis in Leydig cells. Our observation concluded that diosgenin exposure altered steroidogenesis leading into loss of function of germ cells, Sertoli cells and developing spermatozoa, and resulted in defected spermatogenesis.

Herein, we found and discussed on the possible mechanisms and pathways known to be involved in diosgenin related male reproductive toxicity. Our latest findings illustrated new directions to unravel the role of diosgenin in male fertility.

OBJECTIVES

Objectives:

This work aims to evaluate and compare the effects of diosgenin on the spermatogenesis and steroidogenesis in mice:

- Oxidative stress and antioxidant defense activities;
- Sperm and serum hormone analyses; and
- Histopathological and immunohistochemical changes in testes.

MATERIALS AND METHODS

4.1. Selection of animal and maintenance

Twenty five male Swiss albino mice at 56 days old, weighing 25 to 30 g, were selected for this study. They have inbred in the animal house facility, Department of Zoology, Mizoram University, Aizawl, Mizoram. To design a experimental study, the rules and the maintenance of animal was followed likewise the National Guidelines on the Proper Care and Use of Animals in Laboratory research (Indian Science Academy, New Delhi, India).Maximum of 5 animals in each cages were housed in sterilized plastic polypropylene cages well covered with stainless steel grill in a controlled-environment animal room (temperature, 23 ± 1 ºC; relative humidity, 50 ± 10%; photoperiod, 12 h light/dark cycle). Used animal cages were well cleaned and sterilized for further use. There was free access for drinking water and sterilized food (ad libitum) allowed at all through the time. Animal pellet has been provided all throughout experimental study contained adequate amount of carbohydrates, protein, fat, minerals and fibers as suggested by National Institute of Health "Guide for care and Use of laboratory Animals" (NIH production No. 85, 1985:U.S. Bureau of Health, Education and Welfare, Bethesda, MA). The other institute likes Indian Science Academy (ISA), New Delhi, India along with Institutional Animal Ethics Committee of Mizoram University, Mizoram, India (AECMU) were led for investigation for proper maintenance of laboratory animal (**MZU permit number: MZU/IAEC/18/12**). All selected animals were allowed to acclimatize for the inspection and quarantine for 7 days prior to treatment begins.

4.2. Test chemicals

Diosgenin in the form of pure powder was purchased from Sigma Aldrich ($C_{11}H_{10}N_2O_3$, molecular weight 218.21 g/mol, CAS No: 60875-16-3, purity 98%) was obtained from Beijing Zhongnongfa Pharmaceutical Co., Ltd. (Huanggang, China).

4.3. Acute toxicity study for oral exposure of chemical

Before the experimentally planed diosgenin exposure, acute toxicity study was conducted to assess the any clinical symptoms associated prior to its administration. All the experiment was conducted following the guidelines approved by the Mizoram University Institutional Animal Ethics Committee of Mizoram University, Mizoram, India (**MZU permit number: MZU/IAEC/18/12**). This work was approved by both committee for the purpose of "Control and Supervision on Experiment on Animal" and "Ministry of Environment and Forests" New Delhi, India. For testing of acute oral toxicity of chemicals, acute toxicity study was conducted as per the OECD guidelines (OECD Guidelines for the Testing of Chemicals. 2001; 2:12–6) (**Fig. 7**). Fifty adult male Swiss albino mice were selected fororal toxicity study of Diosgenin up to 100, 200, 400 and 600 mg/kg body weight while the control group received vehicle solution only. Animal food in form of pellet and drinking water was freely access all throughout the experiment. Before acute toxicity, all animal were fasted overnight. After single dose of diosgenin exposure, all experimental animalswere clinically monitored for any sigh of dizziness,

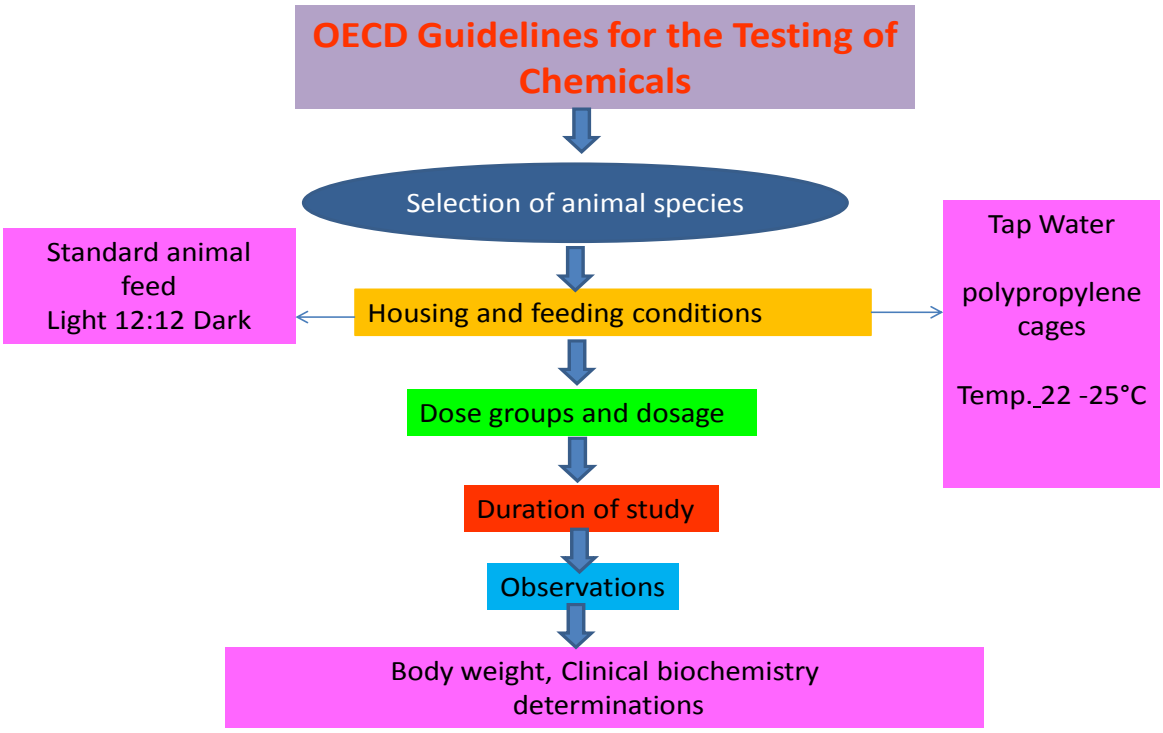


Fig. 7. Procedure of acute toxicity analysis (OECD Guidelines, 2001).

vomiting, diarrhea, bleeding and changed behavior. After continuous observation till four hour, food was provided and monitored further till 14^{th} day. At the 15^{th} day, all mice were kept fasted overnight and scarifies for any toxicity analysis.To estimate the LD_{50} of diosgenin exposure, the method described by Hamilton and Attia, (1978) has been adopted.

4.4. Serum biochemical analysis

At the end of last day of experiment, all mice were kept overnight without food but there was free access for water. Fasted experimental animal were weighed and sacrificed by cervical dislocation under ether anesthesia. All reproductive organs long with kidney and liverwas collected and weighed. Subsequently, for biochemical analysis of hepatic and renal function, serum was collected and kept in -80°C for further evaluation. To evaluate hepatic function, ALP (alkaline phosphates) and SGPT (Serum glutamic pyruvic transaminase) tests were done according to protocol described by Reitman and Frankel, (1957) and Morgenstern *et al.* (1965). Renal test parameters such as serum creatinine and urea level were estimated by the method described by Cheesbrough, (1991) and Gornall *et al.* (1949). To measure the serum cholesterol, triglyceride, LDH and HDL cholesterol level, instruction as described by Cramp, (1973) were followed.

4.5. Dose selection and experimental design

Just before beginning of experiment all animals were weighed and properly watched prior to experiment. They were randomly divided into five groups and each group was containing 5 animals each.

Group1: N (normal control);

Group 2: D10 (received 10 mg/kg/b. w. diosgenin);

Group 3: D50 (received 50 mg/kg b. w. diosgenin);

Group 4: D100 (received 100 mg/kg b. w. diosgenin);

Group 5: D200 (received 200 mg/kg b. w. diosgenin respectively).

Group 1 served as normal control was received only vehicle solution all through the experimental duration (**Table 1**). All diosgenin treated group received diosgenin in vehicle solution (1 mL 20% twin twenty in 100 mL of 70% ethanol). The food and sterile water was freely allowed ad libitum throughout the experimental period. Diosgenin treatments were administered for 40 days (one spermatogenic cycle). All protocols for their use in this investigation are approved by the animal ethics committee (MZUAEC), Mizoram University, Mizoram.

Table 1

Diosgenin doses and experimental design.

Groups	Dose	No. of animals
Group- 1	Control (Normal control)	N = 05
Group- 2	10 mg/kg/b.w Diosgenin	N = 05
Group- 3	50 mg/kg/b.w Diosgenin	N = 05
Group-4	100 mg/kg/b.w Diosgenin	N = 05
Group-5	200 mg/kg/b.w Diosgenin	N = 05

4.6. Sample collection

At the end of last treatment, all mice were kept overnight without food but there was free access for water. Fasted experimental animal were weighed and sacrificed by cervical dislocation under ether anesthesia and blood was collected for serum analysis. Testes and its accessory sex organs such as epididymis, prostate, seminal vesicle and vas deferens were carefully washedin cold PBS, removed the extra adhering fat tissue associated with themand weighed. The gonado – somatic index was calculated by use of the formula; testis mass / final body weight × 100. From each animal right testis and accessory reproductive organ was fixed in Bouin's solution for detailed histopathological and histomorphometric analysis whereas, left side testis and accessory reproductive organs were stored at -80 °C for other oxidative stress and biochemical test.Subsequently, for reproductive serum hormonal status, serum was collected and kept in -80 °C for further evaluation.

4.7. Assessment of reproductive organs weight

After sacrificing experimental animal, testis and its accessory sex organ were dissected out, removed extra fat or adhere tissue, weighed and gonado-somatic index (GSI) was calculated to determine effect of oxidative stress on testis weight [(testicular weight/body weight) × 100].

4.8. Assessment of sperm parameters

The main purpose of sperm quality analysis for any drug is to evaluate the associated side effect of exposed drug to experimental animal. It is very powerful tool in reproductive toxicity research area. Herein, the most common and important parameters are needed to assay are sperm motility, sperm count (caudal and caput concentration), daily sperm production (DSP), sperm morphology, sperm DNA damage and sperm viability. Caudal epididymis was used to assess sperm parameters such as motility, viability, morphology and concentration whereas for daily sperm production in testis is done with testis tissue.

4.8. 1. Sperm motility

Measuring the sperm motility is most initial step of sperm quality analysis that describes the percentage of moving sperm at the time of counting (WHO laboratory manual, 1992). After sacrificing tested animal, caudal epididymis was transacted at the point of origin of the vas deferens at the distal end and immediately wash and kept in watch glass containing 0.5 mL of normal saline (0.9%) maintained at 37 °C. After incubating tissue for a while, the tissue was carefully minced for extrusion of spermatozoa from the caudalepididymis. Any kind of tissue fraction or fatty adhere is carefully removed. Caudal sperm suspension was placed on a clear glass slide and examined the moving spermatozoa under light microscope (Binocular, Olympus CX41, Tokyo, Japan) from different field. Spermatozoa showing any kind of movement considered as motile sperm (MS) whereas, inactive

or sperm with no movement considered as immotile sperm (IM). The number of motile and immotile sperm was quantified and percentage was calculated according to the protocol of WHO laboratory manual, (1992).

Materials and equipments:

1. PBS (pH 7.6)
2. Eppendroff tubes
3. Normal saline solution (0.9%)
4. Clean grease free glass slides and cover slip
5. Fine forceps and
6. Light microscope

Procedure:

a) Add cauda epididymis in 250 µL of PBS and mince it.

b) Place 10 µL of sperm suspension on a clean glass slide and cover with a cover slip and allow it to stand for about a min and examine within 5 min.

c) Encounter 100 spermatozoa from each field under the light microscopeat magnification of 40 × and score motile and immotile sperm from different fields.

d) Examine spermatozoa showing any degree of movement and calculate the % sperm motility.

Motility (%) = Number of motile spermatozoa/ Total number of spermatozoa ×100

4.8.2. Sperm viability

A viability study (percentage of live spermatozoa) was done using eosin/nigrosin stain (WHO laboratory manual, 1992). For sperm viability analysis, supra – vital staining technique was followed to quantify the total number of live and dead sperm. Same epididymal caudal suspension was used to evaluate the percentage of viable or live sperm. A drop of eosin /nigrosin stain is mixed with sperm sample and mixture of that was to prepare an evenly thin film. Further, slide was observed for counting the viable and dead sperm under light microscope (Binocular, Olympus CX41, Tokyo, Japan) at 40 × magnification power and viability was calculated in percentage.

Materials and equipments:

1. Eosin - nigrosin stain
2. Eppendroff tubes
3. Clean grease free glass slides and cover slip
4. Fine forceps and glass rod
5. Light microscope

Procedure:

a) Add caudalsperm suspension on a clear glass slide on which a drop of eosin – nigrosin stain (5% eosin Y + 10% nigrosin; 1:1) is already placed and mix it well with fine glass rod.

b) Take another clean slide and take a drop of mixture and prepare a thin film.

c) Observe the slide under the microscope at 40 × and score hundreds of viable and dead spermatozoa from different field of the slide.
d) Viable sperm remained colorless while non-viable sperm stained red that considered as viable and dead spermatozoa respectively.
e) Sperm viability is calculated as percentage by using the formula as below:

Viability (%) = Number of live spermatozoa/ Total number of spermatozoa×100

4.8.3. Epididymal sperm concentration

Sperm concentration in epididymis is used to upgrade the information about the spermatogenesis in testicular tissue. The method described by Robb *et al.* (1978) has adopted for evaluating the sperm concentration in epididymis (cauda and caput). For counting the spermatozoa, haemocytometer (Fein-optik, Jena, Germany) with improved Neurobauer ruling has applied. Spermicidal solution ($NaHCO_3$; 4g + phenol: 1g in 100 mL distilled water) was 20- fold diluted with sperm suspension and one drop of that mixture was placed on both side of haemocytometer chamber and incubated in wet environment for 30 min. Further, the number of spermatozoa is counted in the four corner squares of the haemocytometer under a under the microscope at 40 × on both side of chamber and average number of spermatozoa is counted.

Materials and equipments:

1. Haemocytometer with improved Neurobauer
2. Spermicidal solution ($NaHCO_3$; 4g + phenol: 1g in 100 mL distilled water)

3. White blood cell pipette
4. Clean grease free cover slip
5. Fine forceps and
6. Light microscope

Procedure:

a) In a white blood cell pipette, suck the sperm suspension up to the 0.5 mark and further the spermicidal solution subsequently to the 11 mark at the top of the bubble chamber.

b) Mix well the above mixture by slowly moving it up and down forward and put one drop on both sides of the humid haemocytometer chambers.

c) Wait for 30 min to allow the spermatozoa settle on chamber.

d) The spermatozoa present in the four corner squares of the haemocytometer chamber will be counted whereas, spermatozoa crossed the lines of the grid should neglected.

e) Count number of spermatozoa at both sides of chambers in haemocytometer under a microscope at 40 × magnification and calculates the average number.

f) The spermatozoa concentration both in cauda and caput was calculated with formula given below.

Sperm concentration is calculated as:

Concentration of spermatozoa = average number of spermatozoa counted (N) × multiple factor (10000) × dilution factor (20)

N × 10000 ×20 spermatozoa

4.8.4. Daily sperm production in testis

To evaluate daily testicular sperm production, the procedure described by Robb *et al.* (1978) has adopted. After removal of upper layer of testis (tunica albuginea), testicular parenchyma was homogenized in 5 mL of 0.9% saline solution together with 20 µL of Triton X – 100. Further, to quantify the elongated spermatids, 1 mL of mixture was diluted with 8.8 mL of distilled water and 200 µL of trypan blue (facilitates spermatids stain). The above prepared sample was placed on haemocytometer and observed for average number of elongated spermatids under light microscope. Elongated spermatids nuclei are generally homogenization resistance at stage 17- 19 of spermatogenesis. Spermatozoa present lie across the outermost lines were not considered for counting whereas those present inside the square were considered for counting. For quantifying daily sperm production, the numbers of spermatids per testis and spermatids per testis were divided by 6.1 days which is the number of days these spermatids are present in the seminiferous tubule through the process of spermatogenesis. The number of spermatozoa counted was expressed as million/mL.

Materials and equipments:

1. Haemocytometer with improved Neurobauer
2. 0.9% saline solution
3. Triton X – 100
4. distilled water

5. Trypan blue
6. Test tubes
7. Clean grease free cover slip
8. Fine forceps and
9. Light microscope

Procedure:

a) Add testicular parenchyma in 5 mL of 0.9% saline solution containing 20 µL of Triton X – 100, and well homogenize it.
b) Take 1 mL of homogenate in another test tube and add 8.8mL of distilled water and 200 µL of trypan blue.
c) Mix the above said solution well by slowly moving it up and down forward and place a drop on both sides of the humid haemocytometer chambers.
d) Wait for a while to allow the spermatids head settle on chamber.
e) The spermatids present in the four corner squares of the haemocytometer chamber will be counted whereas, spermatids crossed the lines of the grid should be neglected.
f) Count the number of spermatids on both sides in the four squares of haemocytometer under a microscope at 40 × magnification and record the average number.
g) The average number of spermatids in both side of chamber will be defined as total sperm number in testis. For daily sperm production, average value of sperm number further divided by 6.1which is number of days spermatids spend in seminiferous epithelium in process of spermatogenesis.

h) Daily sperm production is calculated in million mL $^{-1}$ with following formula given below:

Daily sperm production = Average number of spermatids counted (N) / 6.1 (No. of days spermatids stayed in tubules)

4.8.5. Sperm morphological abnormality

Sperm abnormity is assessed with the caudal sperm smear to investigate the sperm morphological characteristics. The method described by Wyrobek and Bruce, (1975) has adopted to evaluate the sperm abnormality. Caudal suspension was well spread on histological slide, made air dried and stained with eosin. Eosin stains the sperm body that makes easy to observe. Sperm cells have two distinct parts that is head and tail. Abnormality has categorized into head and tail abnormality. There are banana, amorphous and detached head type in head abnormality whereas coil and broken tail type abnormality is considered.Total hundred sperm were encountered at time in different field under a microscope at 40× magnification. The percentage of abnormal and normal sperm with head and tail type were made by counting total number of sperm.

Materials and equipments:

1. Eosin stain
2. Clean grease free glass slides and cover slip
3. Fine forceps and glass rod
4. Light microscope

Procedure:

a) Add caudal sperm suspension on a clear glass slide on which a drop of eosin is placed and well mixed with fine glass rod.
b) Take another clean slide and take a drop of mixture and prepare a thin film.
c) Observe the slide under the microscope at 40× and score hundreds of normal and abnormal spermatozoa from different field of the slide.
d) Type of abnormality is categorized into head abnormality (banana shape, amorphous and detached) and tail abnormality (coil and broken) type.
e) Sperm abnormality is calculated as percentage by using the formula as below;

   Abnormality (%) = Number of abnormal spermatozoa/ Total number of spermatozoa × 100

4.8.6. Sperm DNA evaluation

It is test of DNA integrity and chromatin condensation that used to upgrade the information obtained by Acridine orange (AO) staining of spermatozoa. This cytochemical method of DNA integrity allows the differentiation between double (green fluorescence) and single strand (red fluorescence) DNA because of metachromatic property of AO. Acridine orange is a metachromatic fluorescence probe for demonstration of degree of sperm nuclear DNA susceptibility to in-situ acid-induced denaturation by distinction native double-stranded DNA (green fluorescent, AO^{-}). Same cauda sperm suspension was used for sperm DNA analysis.

There are three type of staining patterns in acridine orange staining for sperm head like green spermatozoa (double –stranded DNA), yellow or red spermatozoa (single –stranded DNA). Normal DNA containing spermatozoa emits green fluorescence light and those damaged DNA containing spermatozoa emits red or orange fluorescence light. An air-dried thin sperm smear were prepared and were fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) for at least one hour and immediately stored at 4 °C. Further, slide was stained by freshly prepared acridine orange and immediately observed using fluorescence microscope (Zeiss Co., Jena, Germany) with 40× magnification and a 460-nm filter (Chohan *et al.*, 2004) and percentage of sperm normal and damaged DNA is determined by counting at least 100 spermatozoa from different field. Notably, it is necessary to observe DNA normality immediately after staining.

Materials and equipments:

1. Acridine Orange (Sigma) solution (0.19 mg/mL in Mcllvain phosphate –citrate buffer (pH=4)
2. Carnoy's solution (methanol/glacial acetic acid, 3:1)
3. Coupling jars and glass beakers
4. Doubled distilled water
5. Clean grease free glass slides and cover slip
6. Fine forceps and glass rod
7. Fluorescence microscope with 40 × objectives and excitation range of 450-490nm.

Procedure:

a) Prepare an air-dried thin sperm smear and fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) for at least one hour or overnight.

b) Wash slide in distilled water and allowed to air dry few min before AO staining.

c) Stain slide in AO solution for 5 min in a covered coupling jar.

d) Gently rinse the slide with distilled water and further mount with distilled water.

e) The percentage of sperm normal and damaged DNA is determined by counting at least 100 spermatozoa from different field at 40 × magnification with excitation of 450nm.

f) Normal DNA containing spermatozoa emits green fluorescence light and those damaged DNA containing spermatozoa emits red or orange fluorescence light.

g) Calculate the average number of damaged sperm DNA and find out percentage of DNA damage.

DNA damage (%) = Number of damaged DNA spermatozoa/ Total number of Spermatozoa × 100

4.9. Estimation of serum LH, FSH, testosterone and estradiol assay

The estimation of serum LH and FSH carried out by the method described by respected DSI convention (RH 152, LOT 122042 and RH 151, LOT 120038). The estimation of serum testosterone hormone was carried out by the method described by human ELISA kit (cat# AA E-1300, Labor Diagnostika Nord, GmbH, Am

Eichenhain, Nodhorn, Germany) whereas for serum estradiol, method described by DiaMetra kit has followed (Ref., DKO003, LOT 4511A). 50 µL of blood serum was added on ELISA well along with standard. Afterward, 100 µL of the enzyme conjugate solution was added into each well and followed with their specific incubation (incubator shaker, model – SLM – INC – OS – 250, Shalimar Spatiality Chemical Ltd., Bangolore, Karnataka, India) period as described by manufactures' instruction (Both testosterone and estradiol have their different incubation time). Subsequently, well content was thrown out and washed three times with washing solution. TMB substrate (tetramethylbenzidine, cat# AA E-0055, Labor Dignostika Nord, GmbH, Am Eichenhain, Nodhorn, Germany) chromogen solution was added to each well and the further incubated in dark for 30 min at room temperature. As a final step of processing, stop solution (100 µL) consist of 0.2 M sulfuric acid were added in all ELISA well and absorbance were recorded using a microplate reader (Spetra Max M2^{e}, micro plate reader, Molecular Devices, Sunny vales, CA, USA) at 450 nm both for testosterone and estradiol.

4.10. Testicular oxidative stress and antioxidant defence activity

Testis tissue was used to evaluate the level of malondialdehyde (MDA, nmol mg-1 protein) by measuring the thiobarbituric acid reactive substance (TBARS) and efficacy of antioxidant defense system**.** To prepare 10% (*w*/*v*) testis homogenate, testis were separated from their outer tunica albuginea and parenchyma was

suspended into ice cold PBS and homogenized in a glass homogenizer, centrifuged for 30 min at 10,000 × g at 4 °C. The supernatant was separated and stored at -80 °C or further biochemical assays.

4.10.1. Determination of testicular malondialdehyde (MDA)

Determination of testicular malondialdehyde (MDA) level is one of the hallmark methods to evaluate testicular function. Higher production of malondialdehyde is related to more per oxidation of polyunsaturated fatty acid, leading degeneration of tissue. Testicular oxidative stress level was measured spectrophotometrically using thiobarbituric acid reactive substances (TBARS), as described by Ohkawa *et al.* (1979). The protein content of the supernatant was determined using the method described by Lowry *et al.* (1951). 10% testis homogenate was prepared in ice cold PBS and further centrifuged in a Eppendroff tubes with 10% TCA (Trichloroacetic acid) at 10000 rpm (for 30 min) at 4 °C and supernatant was carefully separated. Subsequently, the supernatant was mixed with 0.8% TBA (Thiobarbituric acid) in 1:2 ratio (Protein: TBA) and boiled for 45 min till slightly pink color is coming. End product of reaction of all samples were measured against blank (without sample) at absorbance of 540 nm and expressed as nmol of MDA per mg protein.

4.10.2. Quantification of testicular antioxidant profile

To determine antioxidant status of testis tissue, 10% testis homogenate (*w/v*) was used for all biochemical assays by spectrophotometer. To determine testicular superoxide dismutase (SOD) activity, photochemical inhibitionmethod described by of Asada, (1974) was adopted to quantified 50% inhibition of NBT with superoxide dismutase at absorbance of 580 nm. Blank (without sample) was used against all sample by UV – Vis spectrophotometer and activity was expressed as U/mg protein. For estimation of catalase (CAT) activity in the testis tissues, H_2O_2 denegation by catalase enzyme was determined using Aebi *et al.* (1984). This process encounters specific activity of catalase and measured at 240 nm as nmoles of H_2O_2 consumed/min/mg protein. Glutathione S-transferase (GST) activity was estimated as method described by Habig *et al.* (1974). The specific activity of enzyme has been monitored by interaction of 1-chloro-2,4-dinitrobenzene (CDNB) with S-2,4-dinitro-phenyl glutathione (GSH) at 340nm and expressed as U/mg of protein.To evaluate reduced glutathione (GSH) activity in testis, DTNB, 5-5-dithio-bis (2-nitrobenzoic acid) was allowed to interact with sample and absorbance was measured by spectrophotometer at 412 nm (Rahman *et al.*, 2007). Obtained data was calculated from a standard curve and expressed as µmol/mg of protein.

4.11. Histopathological study on the testes

For histopathological and immunohistochemical perspective studies, Bouin's fixed testis were processed through the sequential changes of alcohol (70%, 90%,

90%, 100% and 100%) and cleared with xylene followed with conventional paraffin embedding, sectioned at 5µm. Furthermore, for deparaffinize the histological sections, xylene and sequential changes of alcohol were required. For stability of histological sections, slides were coated with gelatin solution and dried in a hot air oven. Slides were stored in slide boxes and should be used within 3 month as gelatin coating promotes fungal hyphae development.

4.11.1. Histopathology of testis, caudal epididymis, seminal vesicle and adrenal gland

For a comprehensive description of ultrastructure of testis, cauda epididymis, seminal vesicle and adrenal, Bouin's fixed sections were dehydrated in alcohol and xylene, and stained with Hematoxylin and eosin as described by Bancroft and Gamble, (2002). For a detailed view of diosgenin effect on testicular function, same histological stained (Hematoxylin and eosin) slide were used to encounter the potential of sperm production in each group by means of MSTD (mean seminiferous tubule diameter), JTBS (Johnson's testicular biopsy score) and quantification of germ cells and Sertoli cells in each sections. Any kind of detachment in germ cells from its outer periphery membrane, abnormal Sertoli cells, clumps of spermatocytes or spermatogonia, giant cells and dark spottedapoptotic cells with empty spaces were characterized for spermatogenesis assessment. Diameter of seminiferous tubule was measured by MSTD that involve the measuring of testicular lumen by using a micrometric ocular lenses at 40 × magnification under light microscope (Olympus CX41). For each group at least 10 sections were analyzed for measurement.

Seminiferous tubules with normal circular shape were selected and other with irregular shape was neglected for measurement. Average mean of MSTD was calculated and expressed with thickness of µm. For pathological assessment of sperm production, the Johnson's scoring method was adopted (**Table 2**). The production and maturation of epithelium germ cell were appraised by scoring the tubule with a range of 1 – 10. A score of 1 - 4 is suggested for those tubules having only empty lumen, or with only few spermatogonia or spermatocytes, or only Sertoli cells. A score ranging between 5 -7 is suggested for those tubules having only spermatids or few spermatocyteswith no spermatozoa or only spermatogonia, whereas, tubules showing complete set of spermatogenesis or having few spermatozoa with many spermatids or less spermatocytes with many spermatozoa were considered as novel tubule and scored ranges of 8-10. The seminiferous tubules with normal diameter were selected for scoring under light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) at 40 × magnification. For each group at least 10 sections were selected for scoring the tubules. To quantify the germ cells and Sertoli cells in each section, same histological slide stained with H& E were used to examine and quantify the rate of proliferation and maturation of germ cells (spermatogonia, spermatocyte, spermatids and spermatozoa) and number of Sertoli cells per tubule in each group. For quantifying the germ cells, 50% tubules were selected with stages range of I – VIII and rest 50% were selected from IX – XIV to maintain the germ cell variation between different stages of tubule.

Table 2
Johnson's Score (adopted from ***Johnson, 1970***) for assessing spermatogenesis in testicular biopsy.

Score	Description
10	Complete spermatogenesis and perfect tubules
9	Many late spermatids or many spermatozoa present but disorganized spermatogenesis
8	Only a few spermatozoa present
7	No spermatozoa but many spermatids present
6	Only a few spermatids present
5	No spermatozoa or spermatids present but many spermatocytes present
4	Only a few spermatocytes present
3	Only spermatogonia present
2	No germ cells present only sertoli cells (Sertoli cell-only syndrome)
1	No cells

4.11.2. Analysis of Periodic Acid-Schiff-Hematoxylin Stain in testicular tissue

To study the glycogen content in histological sections, PAS staining was performed as method described by Mazaud-Guittot *et al.* (2011). Testis sections were deparaffinise, rehydrated and subsequently oxidized with 0.5% Periodic acid solution. Schiff reagent and hematoxylin were used to counterstained and further dehydrate for mounting. Testicular germinal epithelium glycogen content were carefully observed under light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) at 40 × magnification. For each group at least 10 sections were selected to analyze the glycogen content.

Procedure:

a) Deparaffinise slide with xylene and alcohol (100%, 90%, 70% and 50%) for 10 min and further rehydrate with water.

b) Oxidize sections in 0.5% of Periodic acid solution for 5 min at room temperature.

c) Gently rinse slide in distilled water.

d) Immerse slide in Schiff's reagent for 15 min at room temperature.

e) Gently washed slide in luke warm tap water for 15 min.

f) Further, counterstained by hematoxylin (Harris) for 1min followed with washing in tap water (5 min).

g) Again dehydrate via sequential changes of alcohol and xylene, mount and observe under light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) at 40 × magnification.

4.12. Immunohistochemical studies

All immunohistochemical staining was performed as instruction guideline with slightly modification in protocol. For immunohistochemical analysis, same histological slide were heated in a hot air oven for 10 min at 50 °C. Subsequently, deparaffinization and rehydration was followed by 2 changes in xylene and alcohol gradients at 100%, 90%, 70% and 50% for 10 min. In brief, testis sections were blocked with endogenous peroxidase (3% H_2O_2) and further followed washing with saline phosphate buffer (pH 7.2). Sections were subsequently incubated with diluted normal goat serum for nonspecific binding at room temperature for 30 min, further overnight incubated at 4^0Cwith respected antibody (Santa Cruz Biotechnology). The dilution of primary and secondary antibody for specific protein has been shown in **Table 3**. Incubated sections were gently rinsed in PBS (PH 7.2) followed with secondary incubation with a respective horseradish peroxidase conjugated with streptavidin for 3 hor IgG-HRP for 30 min and then transfer in humid dark environment . Encountered antibodies with respective antibody were exposed with DAB (3, 3?-diaminobenzidine) to detect antigen-antibody complexes. Hematoxylin staining were exposed to all antibodies except PCNA for roughly 1 min and mounted with DPX. Negative control for all respective antibodies was lack of any kind of immunohistochemical expression. Number of positive cells were counted and photographed by counting the presence of total number of antibody positive cells per animal using a light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) at 40 × magnification.

Table 3
Detailed list of primary and secondary antibodies dilution has been used for immunohistochemical analysis.

Protein	Dilution of blocking serum and incubation	Dilution of primary antibody and incubation	Dilution of secondary antibody and incubation	Hematoxylin
PCNA	1:100 µL (30 min)	1:200 (12 h)	1:200 (3 h)	No
StAR	1:100 µL (30 min)	1:200 (12 h)	1:200 (3 h)	Yes
3β-HSD	1:100 µL (30 min)	1:200 (12 h)	1:500 (3 h)	Yes
LH receptor	1:100 µL (30 min)	1:500 (12 h)	1:500 (3 h)	Yes
Aromatase	1:100 µL(30 min)	1:500 (12 h)	1:500 (3 h)	Yes
Bcl-2	1:100 µL (30 min)	1:200 (12 h)	1:200 (3 h)	Yes
Caspase-3	1:100 µL (30 min)	1:200 (12 h)	1:200 (3 h)	Yes

Procedure:

a) Deparaffinise sections with xylene and alcohol (100%, 90%, 70% and 50%) for 10 min and rehydrate to water.

b) Block sections with endogenous peroxidase in an anti-peroxidase solution (40 mL methanol + 2 mL 3% H_2O_2) for 10 min.

c) Gently rinse in distilled water for 5 min.

d) Transfer slide into PBS (PH 7.2) for 10 min and incubate with diluted normal goat serum to block non-specific binding of antibody for 30 min at room temperature.

e) Incubate sections overnight with primary antibody at their specific dilution at 4 ^{0}C.

f) Wash in PBS (primary wash) for 10 min and followed with incubation of their secondary antibody their specific incubation period.

g) For immunoreactivity, expose sections to 0.05% DAB chomogen (3,3-diaminobenzidine) in 0.01% H_2O_2 for 5 min.

h) Counter stain section with hematoxylin staining in required antibody (applied to all antibodies except PCNA).

i) Images were captured from a light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) at 40 × magnification.

4.13. Fertility potential in vivo

To assess the particular drug effect in fertility potential of male, at the end of experiment each diosgenin treated mice were exposed to 5 normal healthy proestrous female mice and allowed to stay with the male prior to last week of treatment. In same manner, normal control male mice were also exposed to normal healthy proestrous female mice as positive control as well. The presence of sperm in female vaginal smear is indication of insemination by male and conform mating and percentage for fertility in each group were calculated. The number of pregnant mice were kept separately and carefully observed though out pregnancy. To assess the fertility potential rate, the number of pregnant mice and their pup's weight immediately recorded as soon as possible and total average number of newborn for each group was calculated (Al-majed *et al.*, 2006).

4.14. Statistical analyses

Statistical analyses for all obtained parameters were subjected to one way ANOVA with Turkey post hoc multiple comparison test (parametric data) using SPSS for Windows (SPSS, Inc. Chicago, IL, USA, ver. 20.0). Alphabet between columns signify that mean ± SEM with different letters considered to be statistically significant at $p < 0.05$ while alphabet with the same letter do not differ significantly at $p < 0.05$. To analyze direct or indirect effect of lipid peroxidation product and comparing with low level of serum testosterone on different parameters, correlation and regression were done. To compare uniqueness and differences ($P < 0.05$) between groups, principal component analysis (PCA) and detrended correspondence analysis (DCA) have been done using PAST (version 1.86 b) software.

RESULTS

5.1. GC-MS profile of diosgenin

The most popular analytical method used for quantification and to reveals the purity of compound via disintegration patterns. GC-MS analysis of diosgenin (Sigma Aldrich) was performed in Central Instumentaion Lab, MZU, Aizawl, Mizoram and spectrum provided by chromatograph shows 98% percentage purity of given compound (**Fig. 8**). The structure of diosgenin is as simillar to cholesterol and many other steroid based hormone. Being a spirostanol saponin, diosgenin contain both hydrophobic aglycone which is linked with hydrophobic sugar moiety. Since, having anactive hydroxyl group (3β position), double bond at C 5,6 and stuructural conformation at position 25insures diosgenin as potantial drug in pharmacology.

5.2. Acute toxicity analysis of diosgenin

The acute toxicity study of diosgenin reveals its minimum toxic effect on body weight and serum biochemical parameters in male Swiss albino mice (N = 10 mice/group) when administered in a single dose of 100, 200, 400 and 600 mg/kg b.w. respectively. Durring 14 day observation period, there was no mortality in all experimental animal including control group received vehical solution. Slightly change in their grooming behaviour and faecal material were noticed within 4 h after exposure of higher doses of diosgenin (400 and 600 mg/kg/b.w.) and behave normal for all activities after some time. There were no reduction in their food and water intake through out experiment thus no significant effect on regular weight gain process. Morever, liver and kindey activity were slightly affected with diosgenin

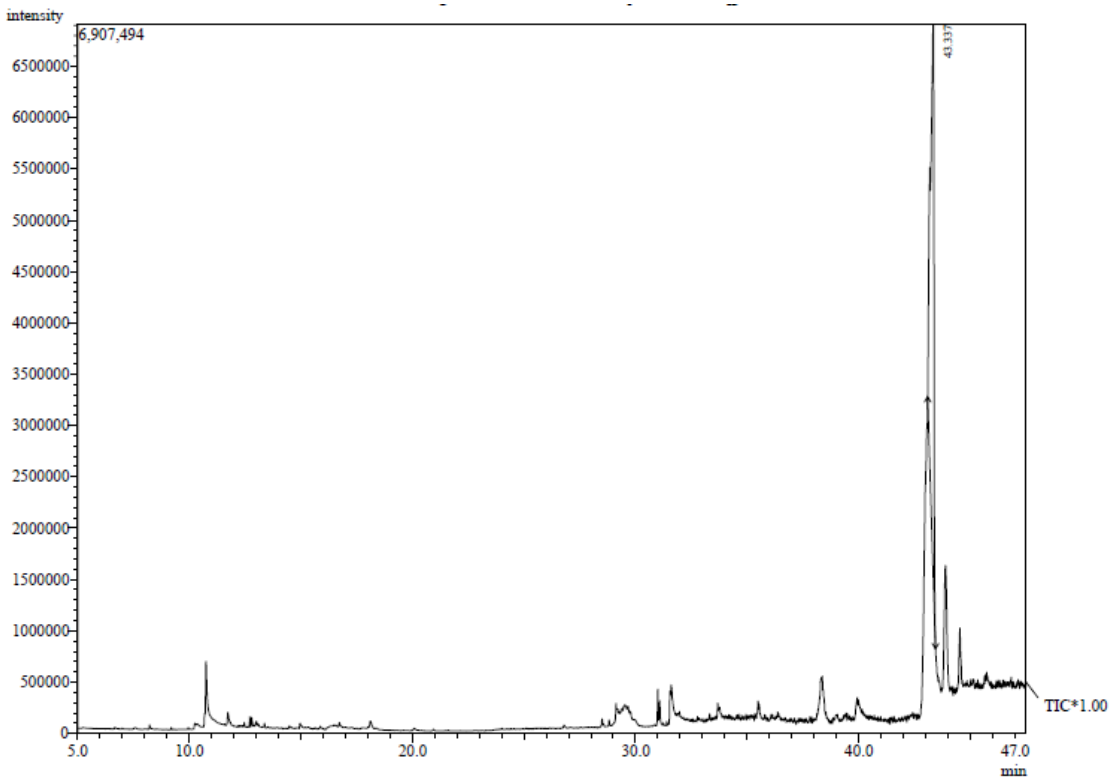


Fig. 8. GC-MS analysis of diosgenin.

exposure when compaired to normal control as shown in **Table 4**. Thus, lethal dose (LD_{50} - mg/kg) was calculated according to Spearman-karber and estimated LD_{50} for diosgenin which is 450.67 mg/kg. Since, diosgenin was found to be safe at all tested doses (up to 600 mg/kg b.w.) and it did not induce any noxious symptom in mice as no mortality was observed during the 3 days of the assessment therefore, 10, 50, 100 and 200 mg/kg body weight diosgenin doses were selected for further in vivo evaluation shown in **Table 2** . As already it is mentioned by Qin *et al.* (2009) that diosgenin is well tolerated in rodents with oral doses of up to～500 mg/kg b.w.

5.3. Diosgenin administration significantly affects body weight and reproductive organs weight

There were a significance great loss in body weight in all diosgenin treated mice whereas, all control (untreated mice) animal gained weight during experimental period (**Table 5**). Percentage of body weight loss (g) among treatment group was found as -5.09 ± 00.15 for D10 mg, -16.39 ± 00.25 for D50 mg, -17.8 ± 00.31 for D100 mg and -22.59 ± 00.44 for D200 mg respectively while control group gained as 12.61 ± 00.12. Moreover, the weight of whole wet liver, kidney and adrenal weight were significantly affected in all diosgenin treated mice in dose dependent manner. The maximum organ weight loss (g) was observed with liver (1403.36 ± 44.39 for N, 1044.90 ± 16.10 for D10, 1154.74 ± 30.44 for D50, and 1163.10 ± 10.08 D100 and 917.30 ± 27.71 for D200) and adrenal weight (13.78 ± 00.93 for N, 8.17 ± 00.22 for D10, 8.86 ± 00.73 for D50, 7.34 ± 00.56 for D100 and 6.58 ± 00.36 for D200).

Table 4

Acute toxicity of diosgenin on body weight and serum biochemical parameters in male Swiss albino mice (N = 5 mice/group).

Parameters	Treatment Groups (mg/kg)				
	N	**D100**	**D200**	**D400**	**D600**
Body weight (mg/kg) Zero day	28.06 ± 0.82a	27.08 ± 0.24a	28.95 ± 0.74a	27.82 ± 0.56a	29.06 ± 0.18a
Body weight (mg/kg) 15th day	28.75 ± 0.12a	28.42 ± 0.14a	2.05 ± 0.52a	28.12 ± 0.15a	29.86 ± 0.05a
Serum SGPT (IU/L)	76.97 ± 0.73a	76.74 ± 0.54a	76.81 ± 0.83a	77.4 ± 0.61a	77.70 ± 0.61a
Serum Creatinine (µmol/L)	0.71 ± 0.05a	0.65 ± 0.07a	0.68 ± 0.06a	0.80 ± 0.06a	0.81 ± 0.11a
Lethal dose (LD_{50} - mg/kg)	**450.67 mg/kg (Spearman-karber estimated LD50)**				

Values are expressed as mean ± standard error mean (n = 5). One way ANOVA with Tukey post hoc multiple comparison test: Different letters within column signify differ significantly at $P < 0.05$ while same letter do not differ at $P < 0.05$. $^{\#}$N- Control. *D – Diosgenin

SGPT- Serum glutamic pyruvic transaminase

Table 5
Effect of diosgenin on body, liver, kidney and adrenal weight in Swiss albino mice.

Parameters	Experimental groups					
	N	**D10**	**D50**	**D100**	**D200**	**F value**
Initial body wt. (g)	30.53 ± 00.51a	31.21 ± 00.32a	31.36 ± 00.28a	29.35 ± 00.17a	30.05 ± 00.45a	5.14
Final body wt.(g)	34.38 ± 00.28a	29.62 ± 00.68b	26.22 ± 00.31b	24.12 ± 01.39b	23.26 ± 01.22b	25.53
% Body wt. gain/loss	12.61 ± 00.12a	-5.09 ± 00.15b	-16.39 ± 00.25c	-17.8 ± 00.31d	-22.59 ± 00.44e	550.14
Liver (g)	1403.36 ± 44.39a	1044.90 ± 16.10b	1154.74 ± 30.44b	1163.10 ± 10.08b	917.30 ± 27.71c	39.87
Kidney (mg)	207.38 ± 05.8a	204.40 ± 04.83a	182.40 ± 05.81b	173.03 ± 07.65b	152.62 ± 03.85b	15.77
Adrenal (mg)	13.78 ± 00.93a	8.17 ± 00.22b	8.86 ± 00.73b	7.34 ± 00.56b	6.58 ± 00.36b	21.35

Values are expressed as mean ± standard error mean (n = 5). One way ANOVA with Tukey post hoc multiple comparison test: Different letters within column signify differ significantly at $P < 0.05$ while same letter do not differ at $P < 0.05$. $^{\#}$N- Control. *D – Diosgenin.

Special attention was given to testis, epididymis, seminal vesicle and prostate weight of diosgenin exposed mice when compared to normal control mice as there was decrease in size and weight shown in **Table 6**. Diosgenin exposed experimental group showed significant ($P < 0.0001$) degeneration of the testis tissue resulted in decline gonadal somatic index (**Fig. 9 A**). Other accessory sex organs such as epididymis, seminal vesicle and prostate show a marked decrease in weight. However, vas deferens was only organ not affected by diosgenin and remains as similar to normal control.

5.4. Diosgenin exposure elevated hepatic, renal and lipid profile

Diosgenin exposure has interfered in liver and kidney activity thus elevated enzyme activity (data shown in **Table 7**). The mean value of serum ALP and SGPT activity significantly ($P < 0.0001$) elevated after diosgenin administration and mean value of serum creatinine and urea level were also altered respectively. The mean value of serum lipid profile level was surprisingly affected with diosgenin as shown in **Table 8**. The serum total cholesterol level was significantly elevated from its normal range at maximum dose (D200) of diosgenin when compare with normal control. Serum triglyceride level was significantly elevated when compared with normal control whereas the level of high density lipoprotein (HDL) and lactate dehydrogenase (LDH) were decreased in dose dependent manner.

Table 6
Effect of diosgenin on testis and its accessory sex organs weight in Swiss albino mice.

Parameters	Experimental groups					
	N	**D10**	**D50**	**D100**	**D200**	**F value**
Testis (mg)	111.94 ± 00.81a	109.52 ± 00.42a	95.42 ± 00.76b	90.25 ± 00.25b	86.75 ± 00.80c	5.089
Epididymis (mg)	59.12 ± 00.86a	48.21 ± 00.54b	47.80 ± 00.71b	40.96 ± 00.53c	36.10 ± 00.27c	15.97
Seminal Vesicle (mg)	215.78 ± 1.70a	193.90 ± 01.24b	189.8 ± 00.88b	148.76 ± 01.60c	76.46 ± 01.89d	23.52
Vas deferens (mg)	29.51 ± 01.01a	20.28 ± 00.87b	20.30 ± 00.95b	17.94 ± 00.75b	15.68 ± 01.05b	21.24
Prostate (mg)	64.96 ± 00.66a	44.05 ± 00.71b	46.98 ± 02.47b	45.62 ± 02.39b	33.26 ± 00.87c	32.38

Values are expressed as mean ± standard error mean (n = 5). One way ANOVA with Tukey post hoc multiple comparison test: Different letters within column signify differ significantly at $P < 0.05$ while same letter do not differ at $P < 0.05$. $^{\#}$N- Control. *D – Diosgenin.

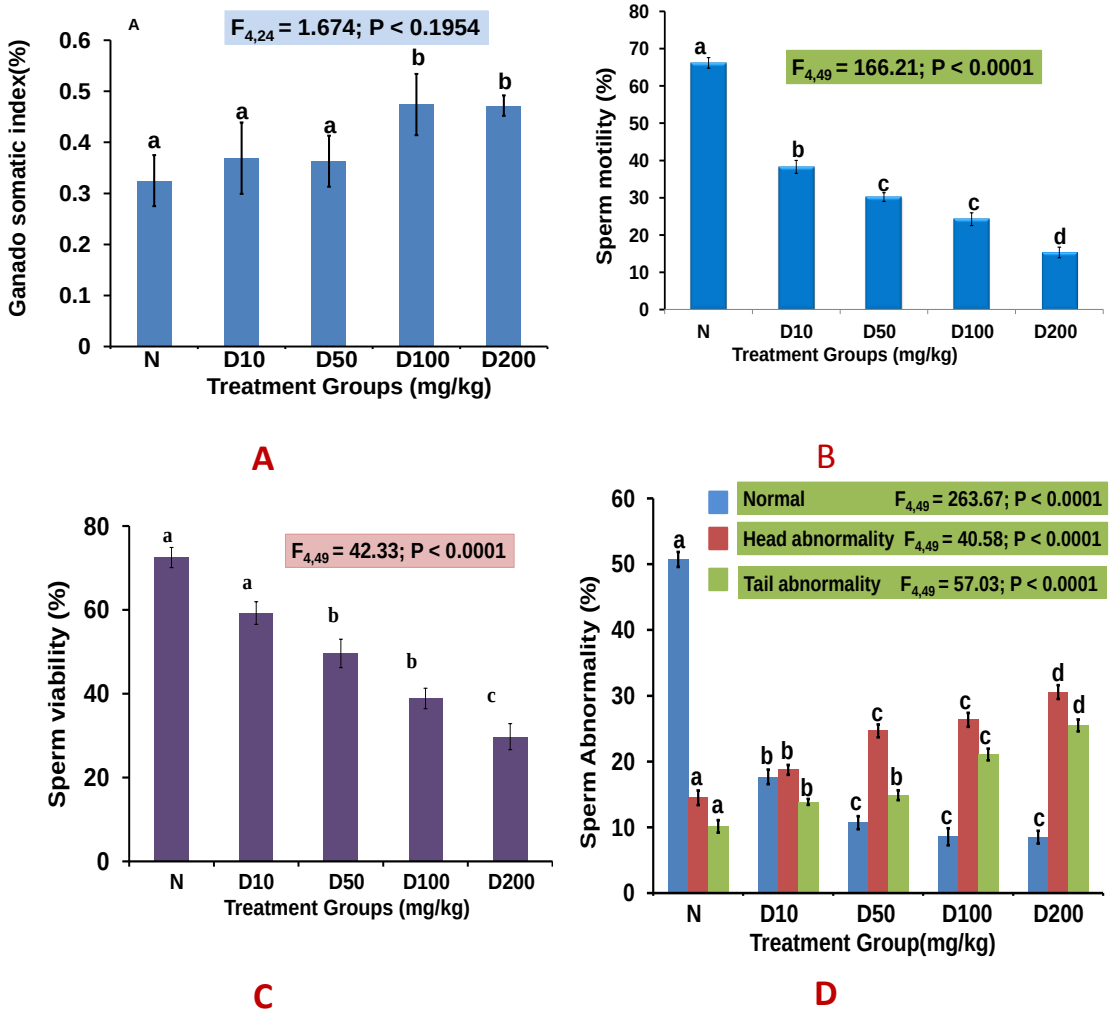


Fig. 9. Effect of diosgenin on gonadal somatic index (A), sperm motility (B), sperm viability (C) and sperm abnormality % (D).

All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar with different letter means statistical significant difference at $p < 0.0001$ and similar letters are not significant.

#N- Control. *D – Diosgenin.

Table 7

Effect of diosgenin on hepatic and renal function assays in Swiss albino mice.

Parameters	Treatment Groups (mg/kg)					
	N	**D10**	**D50**	**D100**	**D200**	**F value**
Serum ALP (IU/L)	140.62 ± 4.43a	151.3 ± 4.32a	158.42 ± 6.24a	190.85 ± 3.90b	201.31 ± 5.87b	27.097
Serum SGPT (IU/L)	153.8 ± 2.16a	158.26 ± 3.43a	166.37 ± 1.14a	185.61 ± 2.16b	188.33 ± 3.12b	38.592
Serum Creatinine (µmol/L)	0.38 ± 0.12a	0.41 ± 0.08a	0.54 ± 0.06a	0.67 ± 0.11a	0.83 ± 0.14b	3.122
Serum Urea	21.71 ± 1.12a	33.21 ± 1.45a	39.46 ± 2.01a	43.89 ± 1.95a	50.66 ± 2.51b	34.656

Values are expressed as mean ± standard error mean (n = 5). One way ANOVA with Tukey post hoc multiple comparison test: Different letters within column signify differ significantly at $P < 0.05$ while same letter do not differ at $P < 0.05$. #N- Control. *D - Diosgenin.

ALP- Alkaline phosphatase**; SGPT-** Serum glutamic pyruvic transaminase

Table 8
Effect of diosgenin exposure on lipid profiles of Swiss albino mice.

Parameters	Treatment Groups (mg/kg)					
	N	**D100**	**D200**	**D400**	**D600**	**F value**
Serum total Cholesterol (mg/dL)	35.75 ± 2.05a	39.2 ± 3.45a	48.13 ± 1.54a	53.62 ± 2.65a	66.81 ± 3.14b	21.581
Serum Triglycerides (mg/dL)	46.88 ± 3.02a	49.36 ± 2.54a	53.2 ± 3.42a	69.53 ± 3.87a	88.07 ± 1.76b	33.093
Serum HDL (mg/dL)	80.54 ± 1.21a	80.05 ± 1.11a	72.95 ± 2.25a	52.31 ± 1.69b	50.54 ± 2.01b	74.666
Serum LDH (mg/dL)	69.28 ± 2.65a	66.98 ± 3.45a	50.68 ± 1.58a	39.78 ± 1.48a	31.56 ± 3.69b	36.642

Values are expressed as mean ± standard error mean (n = 5). One way ANOVA with Tukey post hoc multiple comparison test: Different letters within column signify differ significantly at $P < 0.05$ while same letter do not differ at $P < 0.05$. #N- Control. *D – Diosgenin.

HDL – High-density lipoprotein; **LDH-** Lactate dehydrogenase

5.5. Diosgenin treatment caused impaired sperm parameters

After 40 days of diosgenin exposure, quality of epididymal sperm and testicular sperm production, in the diosgenin-treated groups showed significant ($P < 0.0001$) differences when compared with the control group. The percentage for sperm motility in normal control was 86.2% while diosgenin treated group shows 58.29% in D10, 40.19% in D50, 28.24% in D100 and 25.32% in D200 mg/kg/b.w. respectively (**Fig. 9 B**). The viability study of all diosgenin treated group followed the same trend like motility and shows significant ($P < 0.0001$) decline in percentage of viability (**Fig. 9 C**) in dose dependent manner (78.5% in case of normal control while diosgenin treated group shows 61.25% in D10 mg, 39.62% in D50 mg, 32.87% in D100 mg and 22.75% in D200 mg respectively). There was also significant difference in cauda and caput concentration between the groups (**Fig. 10 A**). Epididymal cauda concentration was exhibited as 31.37×10^6 in case of normal control and shows significantly difference when compared with diosgenin treated groups (26.9 in D10, 19.95×10^6 in D50, 18.67×10^6 in D100, and 18.01×10^6 in D200 mg/kg/b.w. respectively). There was also significant decrease in caput concentration in all diosgenin treated groups when compared to control group (14.82×10^6 in N, 10.1×10^6 in D10, 9.55×10^6 in D50, 8.52×10^6 in D100, and 5.12×10^6 in D200 mg/kg/b.w. respectively. Additionally, sperm morphology characteristics in diosgenin treated groups show significance increase in head abnormality (banana, amorphous and detached head) comparison to tail abnormality (coil/bent and broken) (**Fig. 9 D and 12**). The mean percentage of head abnormality in normal control

group was normal (14.49%) whereas in all diosgenin group there was significant higher level of head anomaly (18.73% in D10, 24.66% in D50, 26.34% in D100 and 30.56% in D200 respectively). An elevated level of tail abnormality (%) was seen in all diosgenin groups when compared to normal control (N - 10.14%, D10 - 13.86%, D50 – 14.88%, D100 – 21.07% and D200 – 25.49% respectively). However, all together abnormality was significant elevated between diosgenin treated mice when compared to normal control group. Moreover, in testicular tissue, there was a significance ($P < 0.0001$) decrease in daily sperm production (DSP) value which is directly affected with low sperm production (**Fig. 10 B**). In diosgenin treated groups, the mean concentration level of testicular sperm production was significant blunted when compared to normal control (N - 10.24×10^6, D10 – 8.06×10^6, D50 – 6.49×10^6, D100 – 6.39×10^6 and D200 – 4.87×10^6 respectively. **Fig. 11** shows the sperm chromatin quality and indicates the percentage of sperm head DNA damage between all groups (N – 2.2%; D10 – 5.67%; D50 – 8.69%, D100 – 9.26% and D200 – 16.07% respectively). Moreover, the **Fig. 12** clearly shows the tendency of increase DNA damaged (red or orange head) spermatozoa in all diosgenin exposed groups when compared to normal control group. However, normal spermatozoa head exhibit green and occurrences of damaged sperm DNA were very less in number.

5.6. Diosgenin altered steroidogenesis by suppressing the LH and FSH concentration

Pituitary hormones such as LH and FSH, stimulate the steroidogenesis as well spermatogenesis were significantly affected by diosgenin administration especially at higher doses as shown in **Fig. 13 A** and **B**. Estimating serum testosterone and

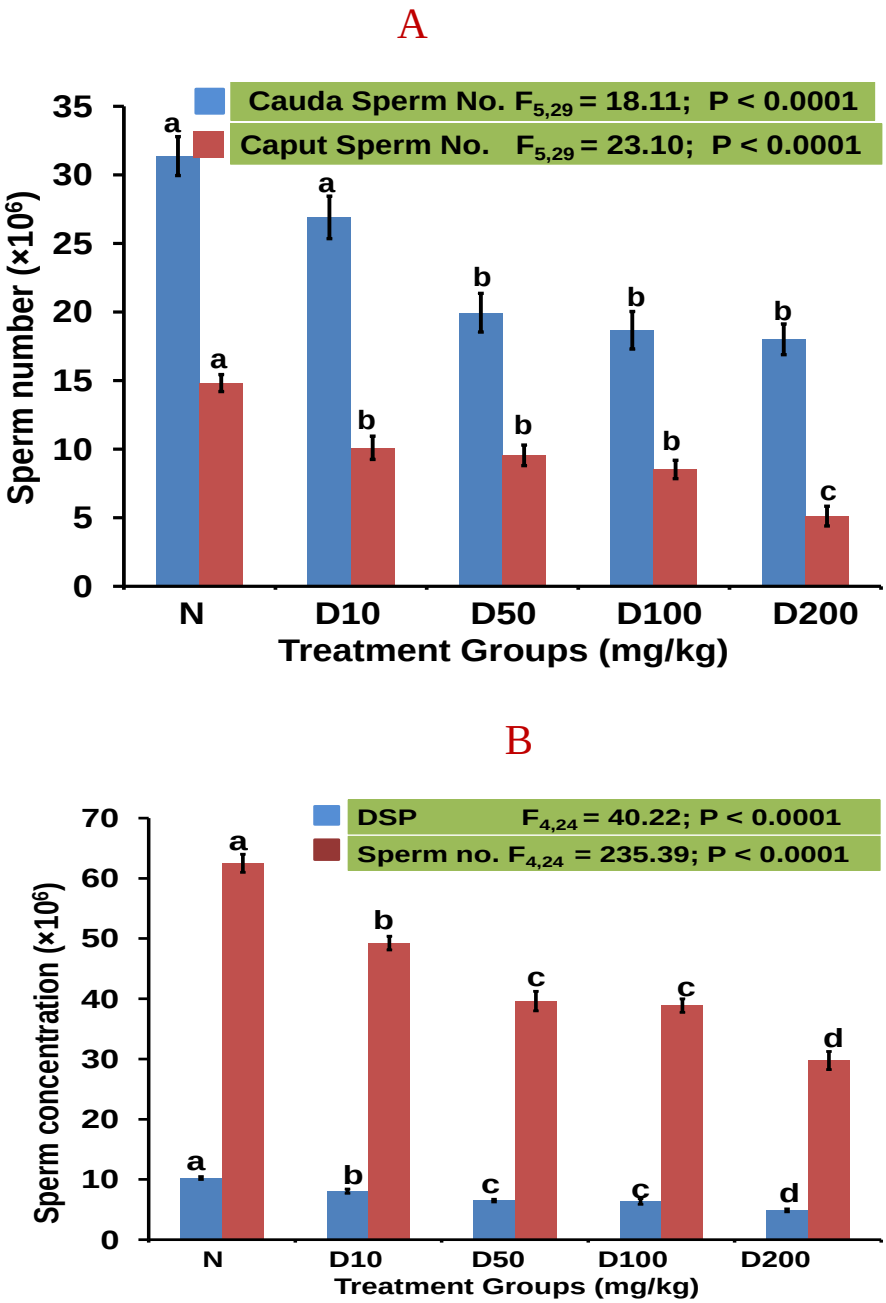


Fig. 10. Effect of diosgenin on epididymal sperm concentration (A) and daily sperm production of male mice (B).

All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar with different letter means statistical significant difference at $p < 0.0001$ and similar letters are not significant.

[#]N- Control. *D – Diosgenin.

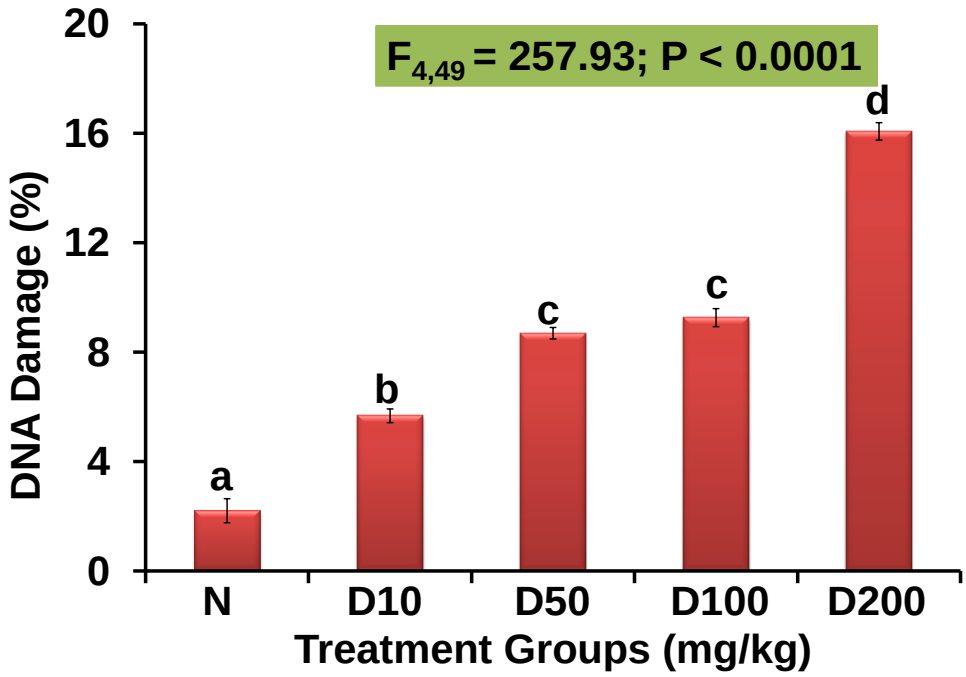


Fig. 11. Effect of diosgenin treatment on sperm DNA damage (%) in male mice. All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar with different letter means statistical significant difference at $p < 0.0001$ and similar letters are not significant.

#N- Control. *D – Diosgenin.

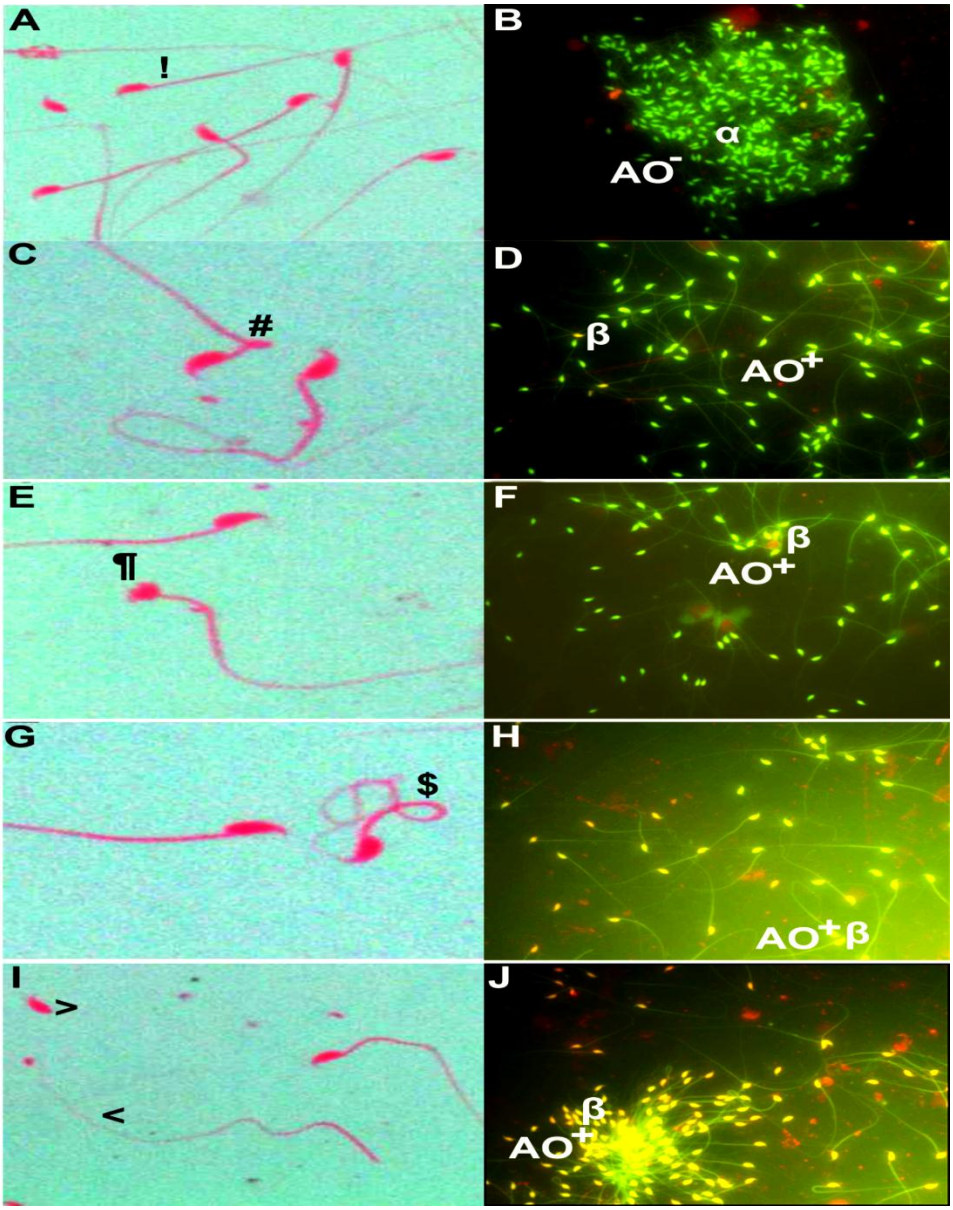


Fig. 12. Effect of diosgenin treatment on sperm morphology characteristics (eosin stain) and sperm DNA damage in male mice (acridine orange test).

Fig. A and B- Normal control; **C and D**- diosgenin 10 mg; **E and F**- diosgenin 50 mg; **G and H**- diosgenin 100 mg; **I and J**- diosgenin 200 mg.

AO$^-$ - Normal native double-stranded DNA (green fluorescent)
AO$^+$ - Denatured single-stranded DNA (orange/red fluorescent)

!- Normal Sperm Head; **#**- Banana Head; **¶**- Amorphous Head; **$**- Coiled Tail; >- Detached Head; <- Broken Tail; **α**- Normal Sperm DNA; **β**- Damaged Sperm DNA

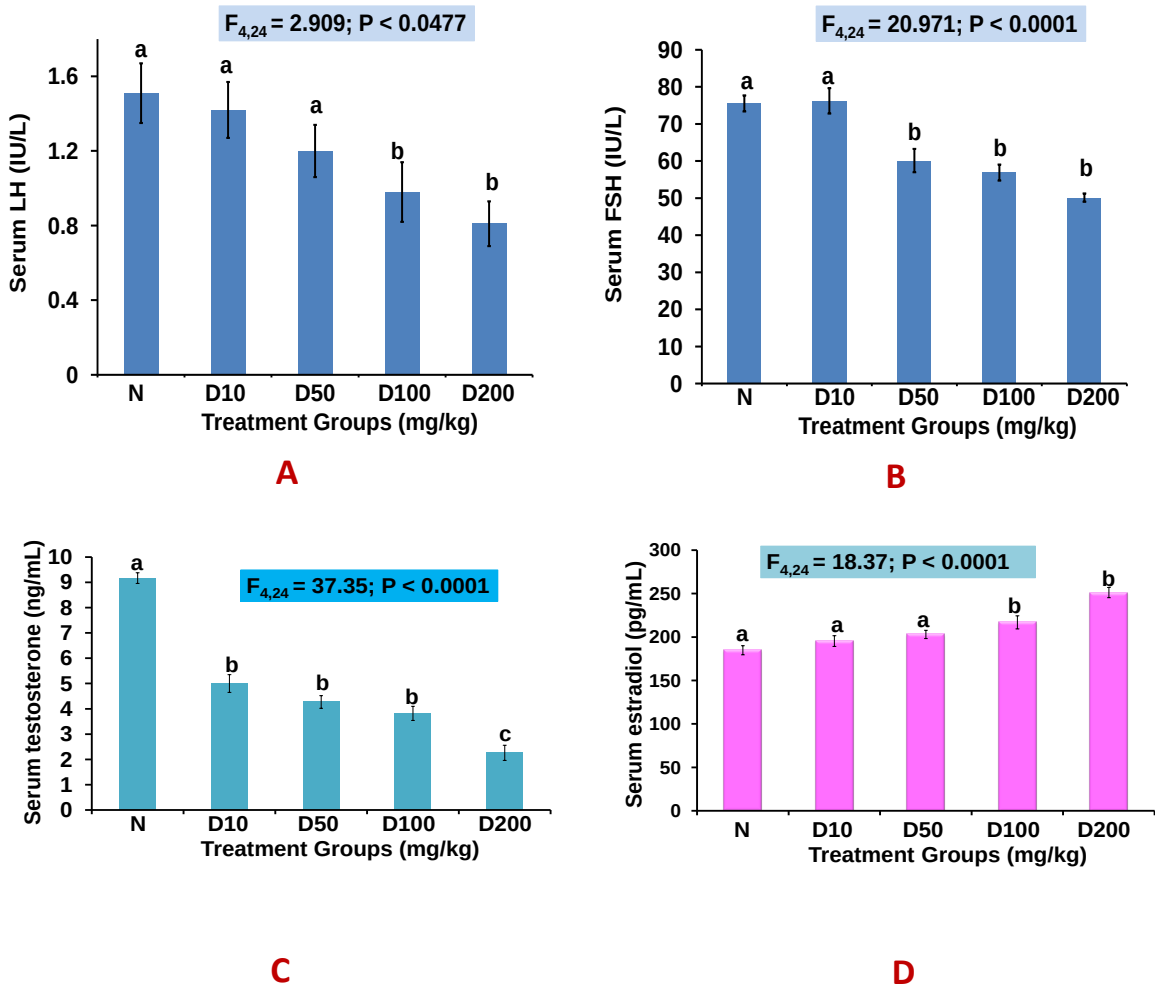


Fig. 13. Effect of diosgenin treatment on serum LH (A), FSH (B), testosterone (C) and estradiol (D) level in male mice.

All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar with different letter means statistical significant difference at p < 0.0001 and similar letters are not significant.

[#]N- Control. *D – Diosgenin.

estradiol verify about functioning of Leydig cell and aromatase activity in testicular tissue. Here, notably, testicular steroidogenesis is significantly affected by diogenin exposure. Our study showed that diosgenin administration in all group reduces serum testosterone level ($p < 0.0001$) together with significant ($p < 0.0001$) increase in estradiol level suggesting the inhibitory role of diosgenin against testosterone production. The mean serum concentration of testosterone in diosgenin treated group was found to be 5 ng/mL, 4.27 ng/mL, 3.82 ng/mL and 2.26 ng/mL in D10, D50, D100 and D200 mg/kg b.w. respectively whereas control group showed as 9.17 ng/mL (**Fig. 13 C**). Moreover, the mean serum level of estradiol was also affected by diosgenin exposure and showed as 195.32pg/mL, 202.96 pg/mL, 216.78 pg/mL and 251.14 pg/mL in D10, D50, D100 and D200 mg/kg b.w. respectively whereas control group showed as 184.75 pg/mL (**Fig. 13 D**). From the above observation, there was clearly a dose dependent effect of diosgenin on testicular steroidogenesis as well pituitary hormone level that affects sex hormone production.

5.7. Diosgenin exposure increases lipid peroxidation product and suppressed the antioxidant defense activity in testicular tissue

Diosgenin administration to normal healthy mice has interfered in testicular oxidative stress marker, malondialdehyde leading into excessive production of free radical species. There was a statically significant ($P < 0.0001$) increase in testicular MDA concentration in all diosgenin exposed experimental animals (**Fig. 14 E**) when compared to the normal control. The concentration of MDA in control

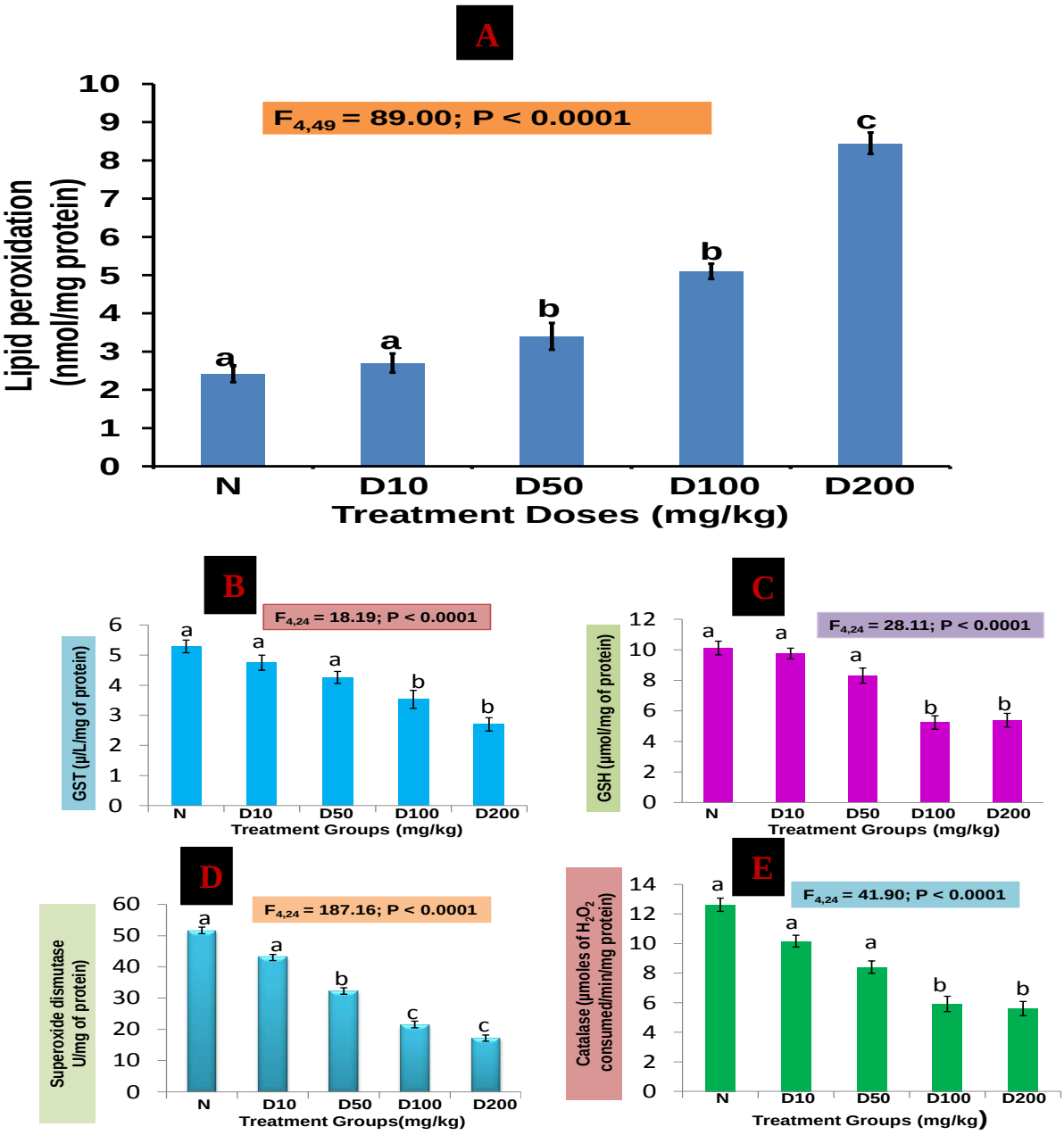


Fig. 14. Effect of diosgenin on testicular MDA content (Fig. A) and antioxidant defense activity (B, C, D and E).

All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar with different letter means statistical significant difference at p < 0.0001 and similar letters are not significant.

[#]N- Control. *D – Diosgenin.

group was showed as 2.41 ± 0.21 nmol/mg of protein whereas diosgenin treated groups exhibited as 2.7 ± 0.25 nmol/mg, 3.4 ± 0.35 nmol/mg, 5.1 ± 0.2 nmol/mg and 8.45 ± 0.28 nmol/mg of protein in D10, D50, D100 and D200 respectively. Notable induced levels of MDA were found with highest dose (D200) of diosgenin.

The effect of diosgenin on testicular antioxidant activity is affected with imbalance of oxidative stress marker (MDA). All experimental animals were receiving diosgenin showed significance ($P < 0.0001$) decreased in testicular SOD activity when compared to normal control group (**Fig. 14B**) suggesting the less efficacy of SOD to dismutase the superoxide anion. The range of SOD in normal control group was showed as 51.6 ± 1.12 U/mg of protein whereas diosgenin treated group exhibited as 43.0 ± 0.97 U/mg, 32.25 ± 1.05 U/mg, 21.5 ± 1.12 U/mg and 17.2 ± 0.99 U/mg of protein in D10, D50, D100 and D200 respectively.

It has been observed that induction of ROS within testicular tissue suppress the catalase activity. In our study we investigated diosgenin supplementation to healthy group significantly ($P < 0.0001$) generates severe oxidative stress (MDA) in testicular tissue and decreases the catalase content which is necessary for healthy spermatogenesis (**Fig. 14 A**). The level of testicular catalase in normal control group was showed as 12.63± 0.45 µmoles whereas diosgenin treated group exhibited as 10.17 ± 0.4 µmoles, 8.41 ± 0.42 µmoles, 5.92 ± 0.52 µmoles and 5.61 ± 0.48 µmoles of H_2O_2 consumed/min/mg protein in D10, D50, D100 and D200 respectively.

GST is susceptible to oxidative stress and excessive production declined its capability to detoxify the free radicals. Glutathione S-transferase (GST) activity in testicular tissue was studied in our study and it revealed that GST was also affected

by oral administration of diosgenin in dose dependent manner. There was significance ($P < 0.0001$) decline in testicular GST content in those group received orally diosgenin at different concentration when compared with normal control group (**Fig. 14 C**).The level of GST in normal control group was showed as 5.29 ± 0.21 µ/L/mg of protein whereas diosgenin treated group exhibited as 4.75 ± 0.25 µ/L/mg, 4.26 ± 0.20 µ/L/mg, 3.53 ± 0.30 µL/mg and 2.70 ± 0.22 µ/L/mg of protein in D10, D50, D100 and D200 respectively.

Glutathione (GSH), a detoxifying agent in antioxidant defense system was assayed and it has been observed in our study that declined in function of other testicular antioxidant enzyme due to intracellular ROS production within testicular tissue leading into decreased GSH activity. Our investigation claimed that there was significant reduction in glutathione content in testicular tissue after oral administration of diosgenin when compared to normal control group (**Fig. 14 D**). The testicular GSH content in normal control group was showed as 10.12 ± 0.45 µmol/mg of protein whereas diosgenin treated groups exhibited as 9.76± 0.35 µmol/mg, 8.31 ± 0.5 µmol/mg, 5.24 ± 0.44 µmol/mg and 5.39 ± 0.45 µmol/mg of protein in D10, D50, D100 and D200 respectively.

5.8. Diosgenin exposure resulted in depletion of tubules and suppressed spermatogenesis

Haematoxyline and eosin stained testicular sections from normal control group and diosgenin treated group were clinically observed and invented the

difference in their histopathological assessment. Normal control group were receiving only vehicle solution does not affect testicular germ cell type and Sertoli cell. **Fig. 15 A** and **B** illustrated control group testicular histology and showed a complete set of spermatogenesis altogether with various stages. All seminiferous tubule were lined with germinal epithelium and surrounded by connective tissue, evenly distributed spermatogonia mother cells between Sertoli cells, large mitotic spermatocytes with lightly stained clumps of chromatin content (primary and secondary), round and elongated spermatocytes below the spermatocytes and clumps of developing spermatozoa attached with Sertoli cells, abundance of spermatozoa in lumen and interstitial cells or Leydig cells located in narrow interstitial spaces of tubules. Overall, normal control groups less or rarely showed detached germ cell from the intact membrane, vacuolated spermatocytes or spermatids and irregular or shrunk seminiferous tubules. Whereas, diosgenin exposed group showed increased tendency of abnormal spermatogenesis consisting of arrest of spermatogenesis, splitting of meiotic spermatogonia from basal region, irregular shaped tubules with wide interstitial spaces, multinucleated giant cells, less Sertoli cells between germ cells, vacuolated spermatocytes, no or less mature spermatozoa in lumen and few number of Leydig cells in interstitial spaces of seminiferous tubules. **Fig. 15 C** and **D** illustrated the light photomicrographs of mice testes parenchyma treated with D10 mg/kg/b.w showed seminiferous tubule with wider interstitial spaces, detached germinal cell from germinal epithelial layer and distorted elongated spermatids. **Fig. 15 E** and **F** illustrated the light photomicrographs of mice testes parenchyma treated with D50 mg/kg/b.w showed shrinkage of seminiferous tubules, thin germinal layer, wide interstitial spaces with few Leydig cell, less number of Sertoli cells between

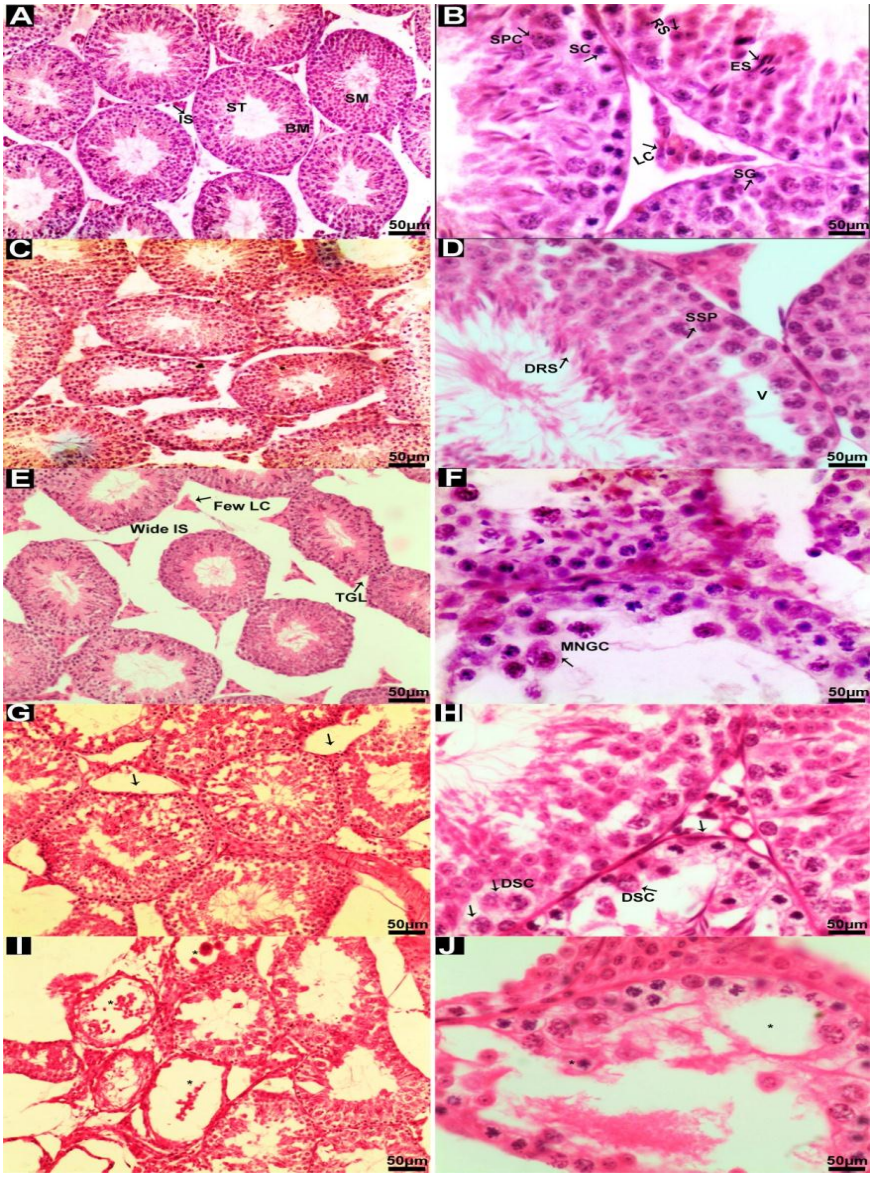


Fig. 15. Effect of diosgenin on testicular ultra structure (Hematoxyline and eosin staining) at ×10 and ×40 magnification.

Fig. A and B shows control group whereas **Fig. C and D**- D10; **Fig. E and F**-D50; **Fig. G and H**- D100; **Fig. I and J**-D200. **(D* diosgenin)**

Seminiferous tubules (ST), Spermatogtonia (SG), Spermatocyte (SPC), interstitial spaces (IS), intact basement membrane (BM), Lumen (LU), sperm mass (SM), Leydig cell (LC), Sertoli cell (SC), elongated spermatids (ES), round spermatids (RS), Distorted spermatids (DRS) and MNGC (multinucleated germ cells).

germinal layer and multinucleate giant cell. **Fig. 15 G** and **H** illustrated the light photomicrographs of mice testes parenchyma treated with D100 mg/kg/b.w showed degeneration of seminiferous tubules, apoptotic spermatogonia and spermatocytes leading into arrest of spermatogenesis. Vacuolization and apoptosis were also seen in Leydig cells situated between the somniferous tubules. **Fig. 15 I** and **J**, illustrated the light photomicrographs of mice testes parenchyma treated with D100 mg/kg/b.w. showed a significant depletion of germ cells, mitotic arrest in secondary spermatocytes, no or less sperm spermatozoa in lumen, many multinucleated giant cell and various necrotic cellular debris in lumen of seminiferous tubule. A wider space between tubules significantly affected the number of Leydig cells which is site of testosterone production. Altogether, it seems that higher dose of diosgenin significantly increases the germ cell loss followed with degenerating spermatocytes and spermatids. Moreover, a trophy of seminiferous tubule also leading into declined in Leydig cells concentration thus affecting testicular steroidogenesis.

For a detailed comprehensive view of spermatogenesis, minutely observation of all seminiferous tubules were done by means of Johnson's testicular biopsy score (JTBS), mean seminiferous tubule diameter (MSTD) and quantification of germ cells and Sertoli cells in each sections. Johnson's testicular biopsy score (JTBS) is scoring method to assess the current status of spermatogenesis by means of identifying its different stages. It revealed about maturity of seminiferous tubule and scored between 1to 10. A normal seminiferous tubule consist of series of cells between spermatogenesis (spermatogonia, spermatocytes, spermatids and spermatozoa) along with Sertoli cells were scored between 10 – 8. However, together with it, there are

many early stages of spermatogenesis which may not consist of some germ cell type or moderate number of germ cells and Sertoli cells in tubule, termed as immature tubule and scored between 7 – 5. A severe affected tubule consists of very few germ cells, Sertoli cells or no cells are termed as defected spermatogenesis or spermatogenesis arrest and scored between 4 – 1. A scored between 1 – 5 may be due to shrinkage of tubule along with increased in lumen area was main cause of poor spermatogenesis. In contrast with above category, we observed spermatogenesis was significantly affected in all diosgenin exposed group when compared with normal control group. The mean Johnson's score in control group was observed as score of 9.8 ± 0.45 whereas diosgenin treated group exhibited as 8.4 ± 0.40, 7.6 ± 0.36, 5.5 ± 0.41 and 4.1 ± 0.33 in D10, D50, D100 and D200 respectively. It seems lower doses of diosgenin (D10 and D50) was less affected compared to higher doses (D100 and D200) as there was only some defected spermatogenesis in seminiferous tubule. **Fig. 16 A** illustrated the outcome of this study.

The mean seminiferous tubule diameter (MSTD) revealed about morphometry of testis section in each group. It explained the diameter and height of tubule to assess the spermatogenesis. In our study, we concluded that normal control group was consist of complete spermatogenesis with regular diameter of 270 ± 5.25µm and whereas in all diosgenin exposed groups showed a declined diameter as 235 ± 7.35 µm, 209 ± 6.83 µm, 180 ± 5.47 µm and 157 ± 5.2 µm in D10, D50, D100 and D200 respectively. The findings of this parameter are illustrated in **Fig. 16 B**.

The number of germ cells and Sertoli cells per tubule in testis of normal and diosgenin treated group were assessed and percentage was calculated to compare the

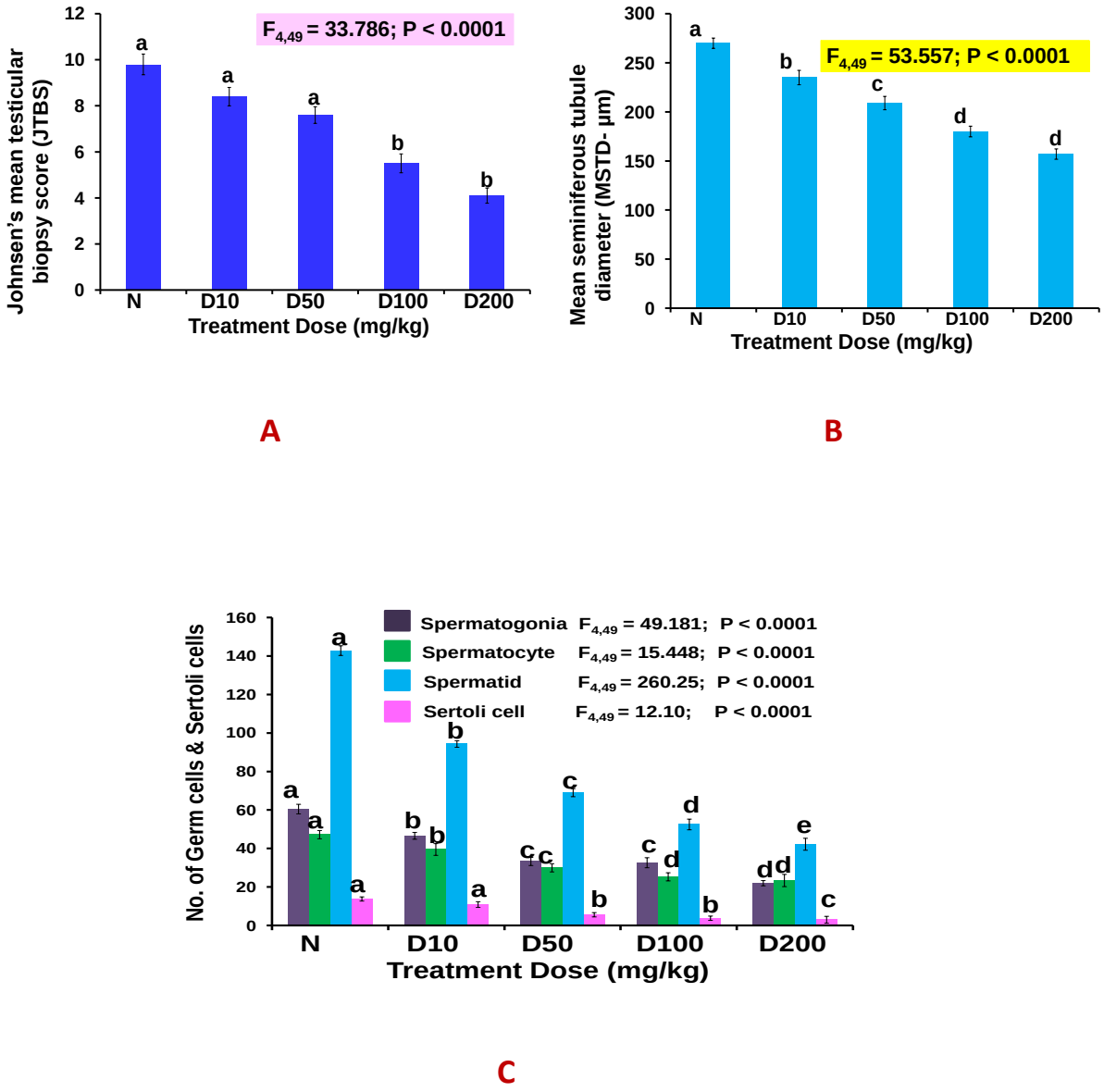


Fig. 16. Effect of diosgenin on Johnson's mean testicular biopsy score (A), seminiferous tubule diameter (B) and number of germ cells and Sertoli cells (C).

All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar with different letter means statistical significant difference at p < 0.0001 and similar letters are not significant.

#N- Control. *D – Diosgenin.

seminiferous tubule cell count between groups. In control group, seminiferous tubule was consisting of abundance of germ cells (60.5 ± 2.5), spermatocytes (47.2 ± 2.12), spermatids (142.8 ± 2.5) and Sertoli cells (13.8 ± 1.1). However, diosgenin exposure significantly declined the number of germ cell and Sertoli cell in seminiferous tubule. All seminiferous tubule cell including spermatogonia, spermatocytes, spermatids and spermatozoa are completely dependent for their nutrition on Sertoli cells and loss of it directly affect the spermatogenesis and maturation of sperm. The Sertoli cells, nurse cell of seminiferous tubule, were seriously affected in seminiferous tubule when compared with normal control group (10.9 ± 1.5, 5.6 ± 1.14, 3.8 ± 1.08 and 3.0 ± 1.0 in D10, D50, D100 and D200 respectively). Spermatogonia, most sensitive and initiator of spermatogenesis, were mostly affected with diosgenin exposure (46.6 ± 1.8, 33.3 ± 2.1, 32.6 ± 2.6 and 22 ± 1.33 in D10, D50, D100 and D200 respectively). Interestingly, declined in germ cell concentration further affected mean proliferation rate and the number of spermatocytes (39.5 ± 3.03, 30 ± 2.12, 25.3 ± 2.15 and 23.4 ± 3.15 in D10, D50, D100 and D200 respectively) and spermatids (94.3 ± 1.7, 69 ± 2.1, 52.5 ± 2.8 and 42.2 ± 3.1 in D10, D50, D100 and D200 respectively) in testicular tubule. **Fig. 16 C** illustrated the finding of this result.

5.9. Diosgenin administration and glycogen content in testicular tissue

A histochemical analysis to assess the glycogen content in testicular tissue reveal about the carbohydrate concentration in cells of seminiferous tubule. PAS staining of testis from normal control group showed dark and bright staining in

spermatogonia and spermatocytes altogether with lipid content in their cytoplasm (**Fig. 17**). Sertoli cells, a major storage for carbohydrate to nourish other spermatogenesis cell, showed abundance of glycogen granules with adequate amount of lipid molecule. In comparison to normal control group, diosgenin treated group showed a slightly blurred color spermatogonia and Sertoli cells in lower doses of diosgenin (D10 and D50). Whereas, spermatocytes and spermatids were not stained with PAS staining when compared with spermatogonia. Higher doses of diosgenin (D100 and D200) significantly affected the glycogen content in all cells present in spermatogenesis sequence. The most affected cell was noticed as spermatogonia as there was serious reduction in carbohydrate and fat ratio in outer germ layer. Sertoli cells in higher doses were noticed with shrinkage cytoplasm thus affecting the balance between lipid molecules and glycogen amount.

5.10. Histology of cauda, seminal vesicle and adrenal

A cauda epididymis histological analysis in diosgenin treated group revealed the columnar epithelium and sperm mass difference when compared to normal control group (**Fig.18**). Normal control group showed large round tubules with outer thin layer of columnar pseudostratified epithelium and inside lined by stereocilia or microvilli. There was abundance of sperm mass in the lumen of cauda lumen. Diosgenin exposed groups significantly affected the cauda tissue as there was decreased in size of tubules with disorganized columnar epithelium cell and noticeable loss in microvilli. Many of tubules were noticed with few or no sperm density in higher doses of diosgenin treated group. However, lower doses of

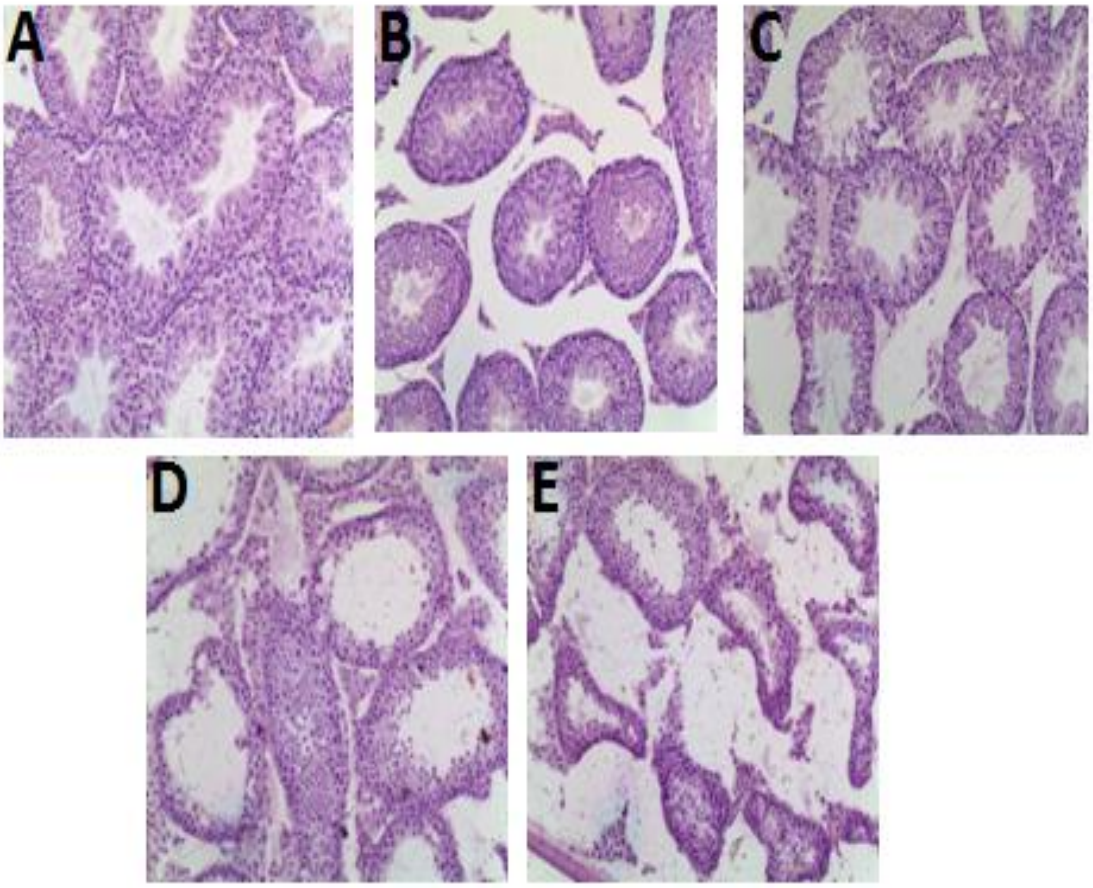


Fig. 17. Effect of diosgenin on glycogen content in testicular tissue (Periodic Acid-Schiff-Hematoxylin, 10×).

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg and **E**-diosgenin 200 mg

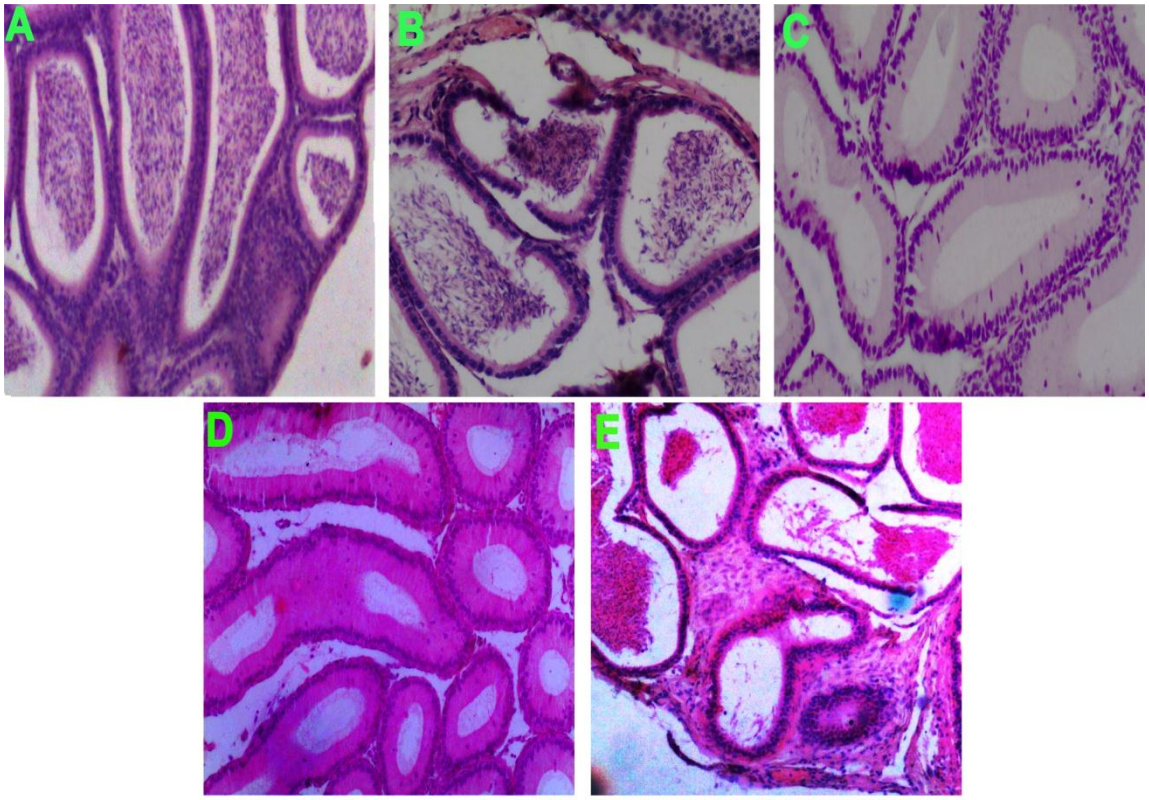


Fig. 18. Effect of diosgenin exposure on cauda epididymis ultra structure of male mice (Hematoxyline and eosin staining) at 10 × magnification.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg and **E**-diosgenin 200 mg

diosgenin showed no noticeable difference in overall size of epithelial cells but sperm mass was only affected.

Another most sensitive parameter to investigate drug (diosgenin) effect on spermatogenesis, seminal vesicle is perfect site to look out its histology for any kind of alteration. To investigate the diosgenin effect on seminal vesicle, histological analysis of seminal vesicle were done due to its direct relation with sperm storage, nutrition and maturation. Normal histology of seminal vesicle consists of outer thin layer of columnar pseudo stratified epithelial cells with smooth muscles wall, villous mucosa and much finger-like projection towards lumen. The lumen of seminal vesicle was randomly occupied by microvilli and many secretary glands. However, in our study we observed that diosgenin significantly affected the seminal vesicle tissue as there was noticeable shrinkage in outer columnar cells along with decreased lumen area (**Fig. 19**). There was reduction in glandular cells in villous mucosa layer resulted in less mature sperm in cauda epididymis thus increase the tendency of sperm morphological abnormalities in all diosgenin treated group when compared to normal control group.

A detailed histological analysis of adrenal was done and found noticeable changes in cortex and medulla area. Herein, we observed a decline in area of adrenocortical region with increased in medullar region after diosgenin treatment (**Fig. 20**). In another hands, normal control group showed with a balance differentiation of cortical and medullar region. It is suggested that changes in steroidogenic tissue of adrenal due to increased oxidative stress leading a damaging effect.

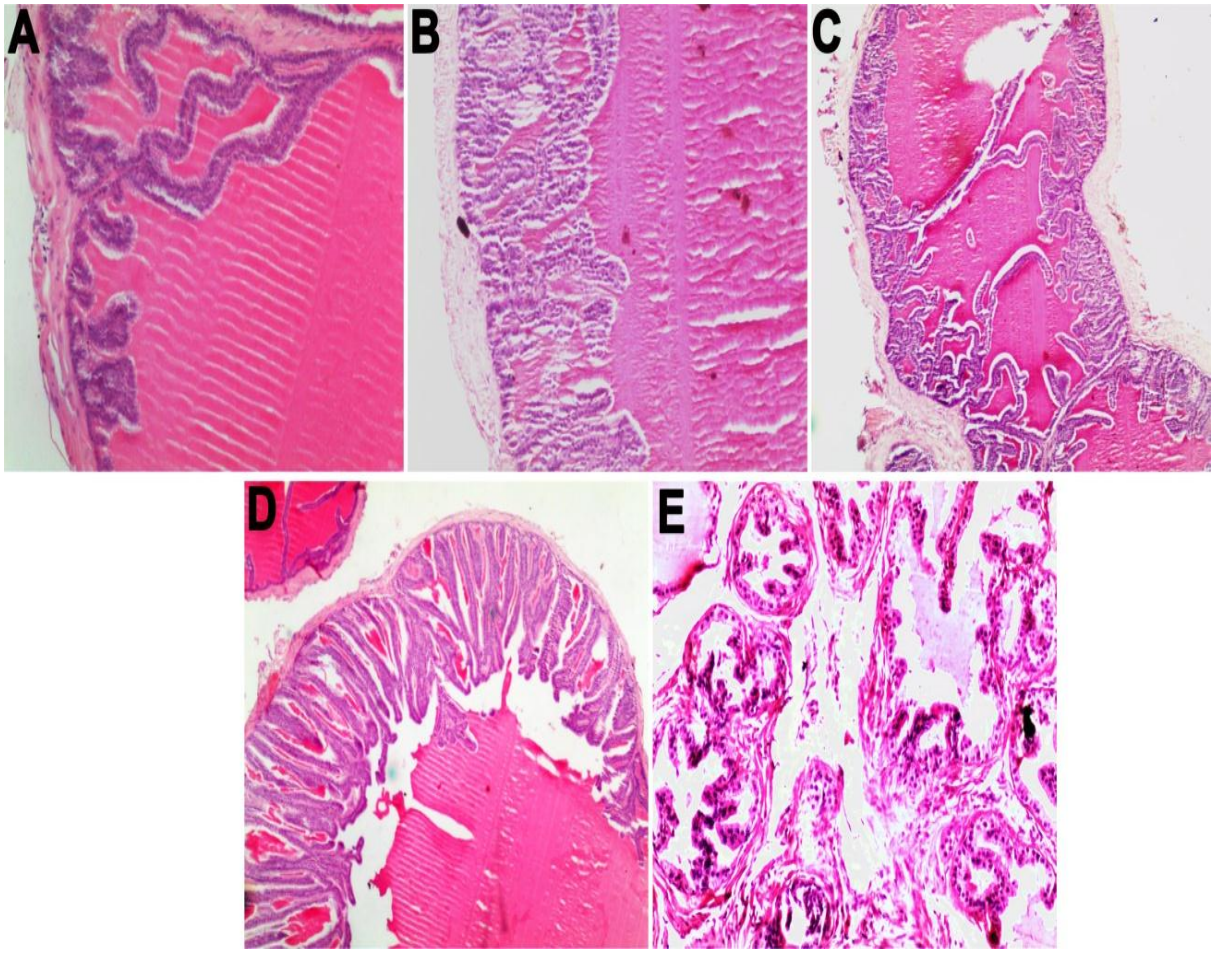


Fig. 19. Effect of diosgenin exposure on seminal vesicle ultra structure of male mice (Hematoxyline and eosin staining) at 10 × magnification.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg and **E**-diosgenin 200 mg

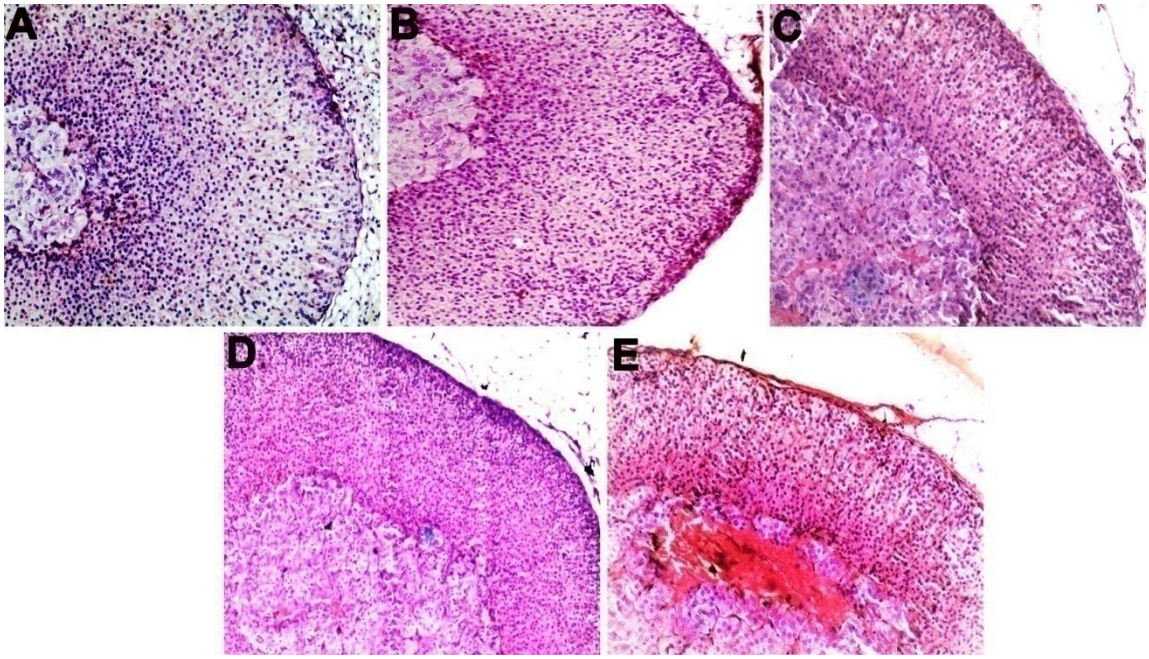


Fig. 20. Effect of diosgenin exposure on adrenal ultra structure of male mice (Hematoxyline and eosin staining) at 10 × magnification.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg and **E**-diosgenin 200 mg

5.11. Diosgenin downregulated the expression of PCNA, StAR, 3β-HSD, LH receptor and upregulated the aromatase, Bcl-2 and caspase-3 in testicular cells

PCNA (proliferating cell nuclear antigen), a potent biomarker for proliferation in spermatogonia and spermatocytes (leptotene, zygotene and pachytene spermatocytes) has been extensively used for to immunohistochemical studies (Jaskulski*et al.*, 1998; Celis*et al.*, 1987; Kang *et al.*, 1997). Now days, it has been used as an early biomarker for detecting chemically induced testicular toxicity due to its DNA repair nature. In our study, we observed that diosgenin exposure in normal healthy mice significantly affected expression of PCNA protein in germ cells and spermatocytes of seminiferous tubule (**Fig. 21**). In another side, normal control group showed well expression of PCNA in nuclei of outer germinal layer (spermatogonia). Moreover, some staining in spermatocytes (prophase I) was also noticed in their nuclear content however, other cells involved in spermatogenesis like spermatids and spermatozoa were devoid of its expression. Herein, the immunostaining density of PCNA (mean gray value) in normal control group was recorded as 138.17 ± 8.66 whereas in diosgenin treated group it showed as 138.86 ± 8.26, 131.11 ± 6.3, 112.66 ± 5.17 and 93.5 ± 4.13 in D10, D50, D100 and D200 respectively ($F_{4,24} = 8.40$; $P < 0.0004$).

In testicular tissue, StAR (steroidogenic acute regulatory protein) is one of the most important enzymes in steroidogenic pathway controls cholesterol transfer to inner mitochondrial membrane through a series of event. It is primarily located in Leydig cells situated between interstitial spaces of seminiferous tubules and on head

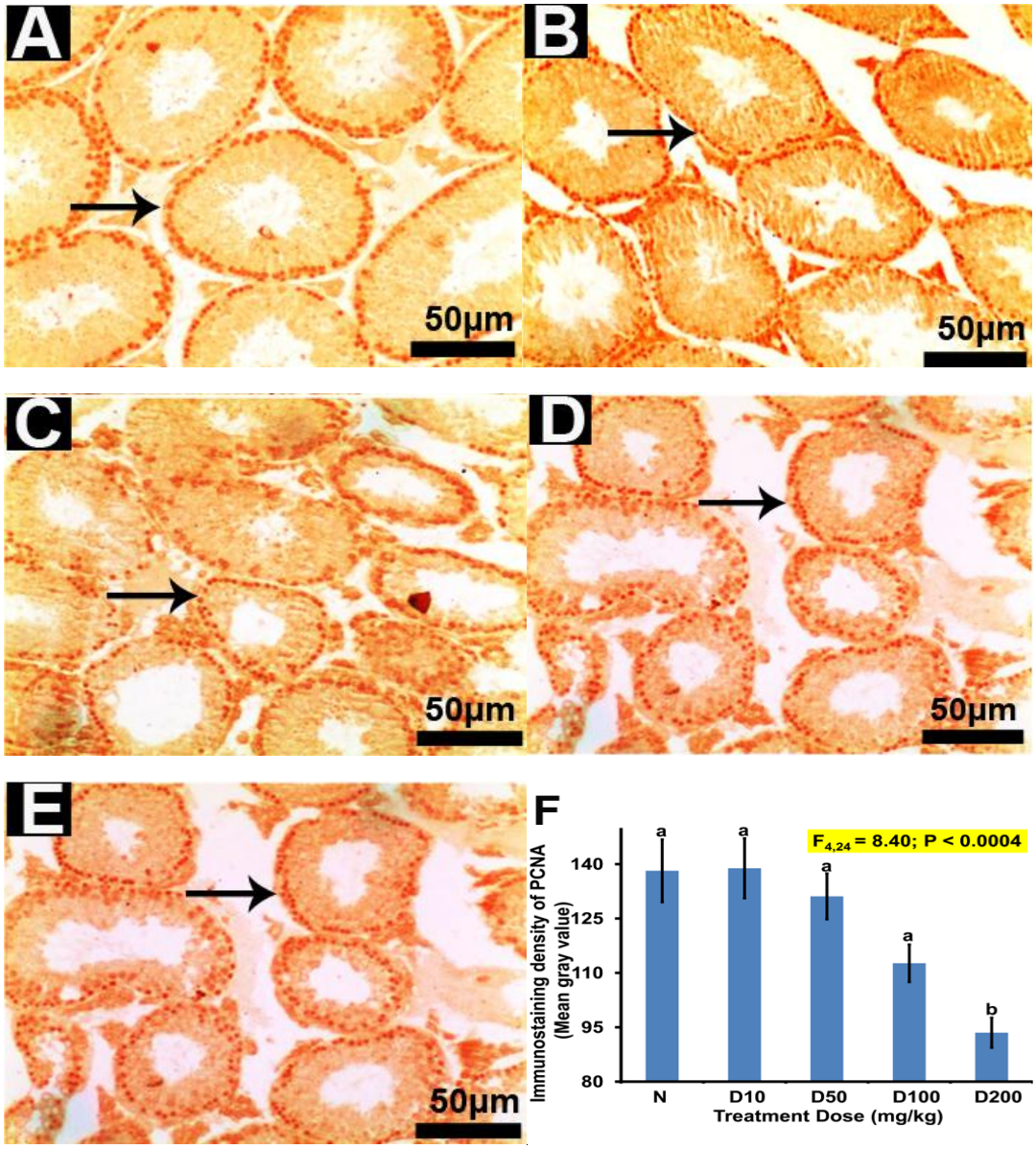


Fig. 21. Effect of diosgenin on PCNA expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of PCNA

#N- Control. *D – Diosgenin.

of developing spermatozoa. The expression of StAR is dependent on the concentration of Leydig cells. Like other steroidogenic enzyme involved in steroidogenesis, StAR is also affected with exogenous steroids. In this study, we revealed that the expression of StAR in testicular Leydig cells was seriously affected with diosgenin treatment when compared to normal control (**Fig. 22**). The immunohistochemical study of StAR in normal control group showed a positive expression in Leydig cells and in sperm masses towards lumen. In addition to that, it is concluded that higher doses of diosgenin was seriously declined the StAR expression however, the lower doses were not that much effective than compared to normal control group. Herein, the immunostaining density of StAR (mean gray value) in normal control group was observed as 196.37 ± 13.51 whereas in diosgenin treated groups it showed as 183.92 ± 13.02, 142.87 ± 11.03, 112.21 ± 5.61 and 111.37 ± 5.58 in D10, D50, D100 and D200 respectively ($F_{4,24} = 11.22$; $P < 0.0001$).

3β-hydroxysteroid dehydrogenase (3β-HSD) is crucial enzyme in process of steroidogenesis that catalyzes the steroid into progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone from pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA) and androstenediol respectively. It is generally well located in gonads (testis and ovary) and cortical part of adrenal gland therefore involved in production of sex hormone and corticosteroids (glucocorticoids and mineralocorticoids) respectively. In mouse testis, immune histochemistry study revealed its expression in Leydig cells however in many other primate studies revealed its expression in Sertoli cells. It is well documented that expression of 3β-HSD is regulated by LH receptor hence activation of LH receptor

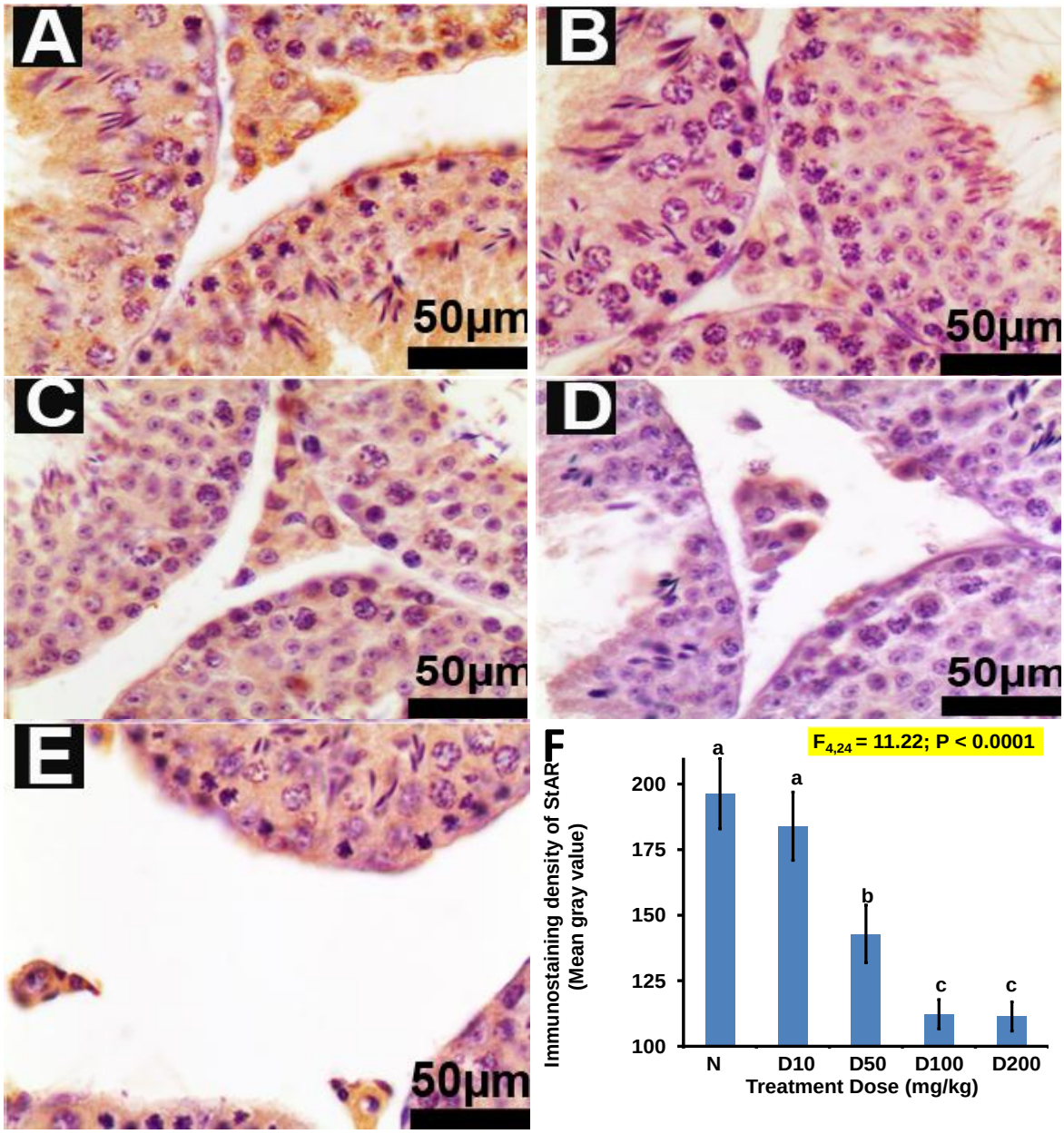


Fig. 22. Effect of diosgenin on StAR expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of StAR

#N- Control. *D – Diosgenin.

stimulates more catalization of steroids into sex hormones and corticosteroids through steroidogenesis. Since LH secretion from anterior pituitary is necessary for stimulation of enzymes involved in steroid production therefore LH receptor plays a regulatory control over 3β-HSD expressions. In normal control group, the majority of 3β-HSD expression was documented in Leydig cells and rarely seen in other cells of seminiferous tubules. An interrupted steroidogenesis has been documented after diosgenin treatment to healthy normal mice as there was decline in expression of 3β-HSD testicular tissue (**Fig. 23**). In higher doses of diosgenin (D100 and D200), 3β-HSD expressions were significantly reduced compared to lower doses of diosgenin (D10 and D50). Herein, the immunostaining density of 3β-HSD (mean gray value) in normal control group was recorded as 195.76 ± 7.4 whereas in diosgenin treated groups it showed as 187.36 ± 6.79, 176.38 ± 6.24, 151.42 ± 5.22 and 142.12 ± 5.87in D10, D50, D100 and D200 respectively ($F_{4, 24} = 13.19$; $P < 0.0001$).

LH receptors (LHR) or human chorionic gonadotropin (hCG) is well located in gonads (testis and ovary) and their accessory sex organs. Besides that, LHR expressions are also reported in brain and placental tissue. LHR plays a major role in Leydig cells development and differentiation in the early phases of testicular cells development necessary for steroidogenesis. It shows regulatory control on hypothalamic pituitary gonadal (HPG) axis through high-affinity G protein coupled receptors (GPCRs). Moreover, in testis, cAMP independent messengers regulates activation of LHR, leading a controlled over steroidogenesis in Leydig cells. It is already reported in many study that LHR is highly susceptible to exogenous steroid present in numerous food leading into decline in altered steroidogenesis. In our

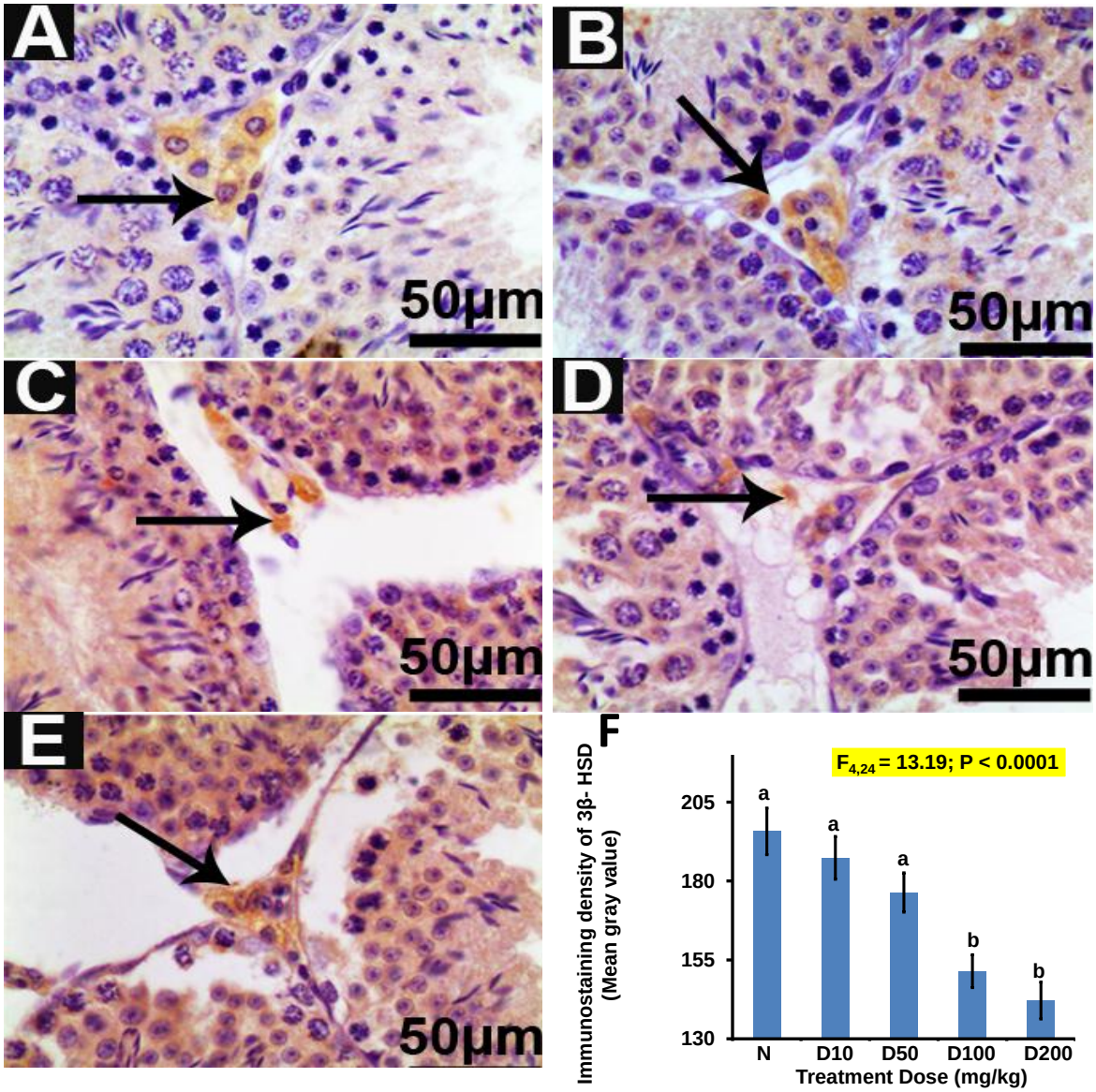


Fig. 23. Effect of diosgenin on 3β-HSD expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of 3-βHSD

#N- Control. *D – Diosgenin.

study, we claimed that diosgenin being a steroid compound, altered the expression of LHR either via interfering in binding ability with steroid receptors or by various unknown mechanism. We observed that normal control group consist of a massive density of LHR positive stained Leydig cells in interstitial spaces of seminiferous tubules. However, diosgenin treatment to normal healthy mice significantly declined in LHR expression in dose dependent manner when compared to normal control group (**Fig. 24**). Herein, the immunostaining density of LHR (Mean gray value) in normal control group was recorded as 195.68 ± 8.27 whereas in diosgenin treated group it showed as 160.98 ± 7.35, 157.61 ± 7.16, 157.2 ± 7.35 and 152.35± 7.72 in D10, D50, D100 and D200 respectively ($F_{4,\ 24} = 5.36$; $P < 0.0042$).

Aromatase (P450) also called as CYP19 is main enzyme in steroidogenesis that involved in aromatization of testosterone into estradiol through. The aromatase P450 enzyme is well localized in cell type of seminiferous tubules however; prime location of this enzyme is reported in Leydig cells and Sertoli cells. Some early phases of germ cells were also reported for aromatase expression. Furthermore, the expression of aromatase P450 is modulated by estrogen receptors via cell signaling. It is well documented that male testis needs both testosterone and estradiol for healthy spermatogenesis. Like other enzyme involved in steroidogenesis, aromatase is also sensitive to many exogenous steroid and environmental circumstances as altered aromatase activity contributed to the high estradiol, could cause serious fertility issue in male. In this study, the immune staining revealed that there was exclusively increased in aromatase expression in testicular Leydig cells after diosgenin treatment (**Fig. 25**) contributing to elevated level of serum estradiol

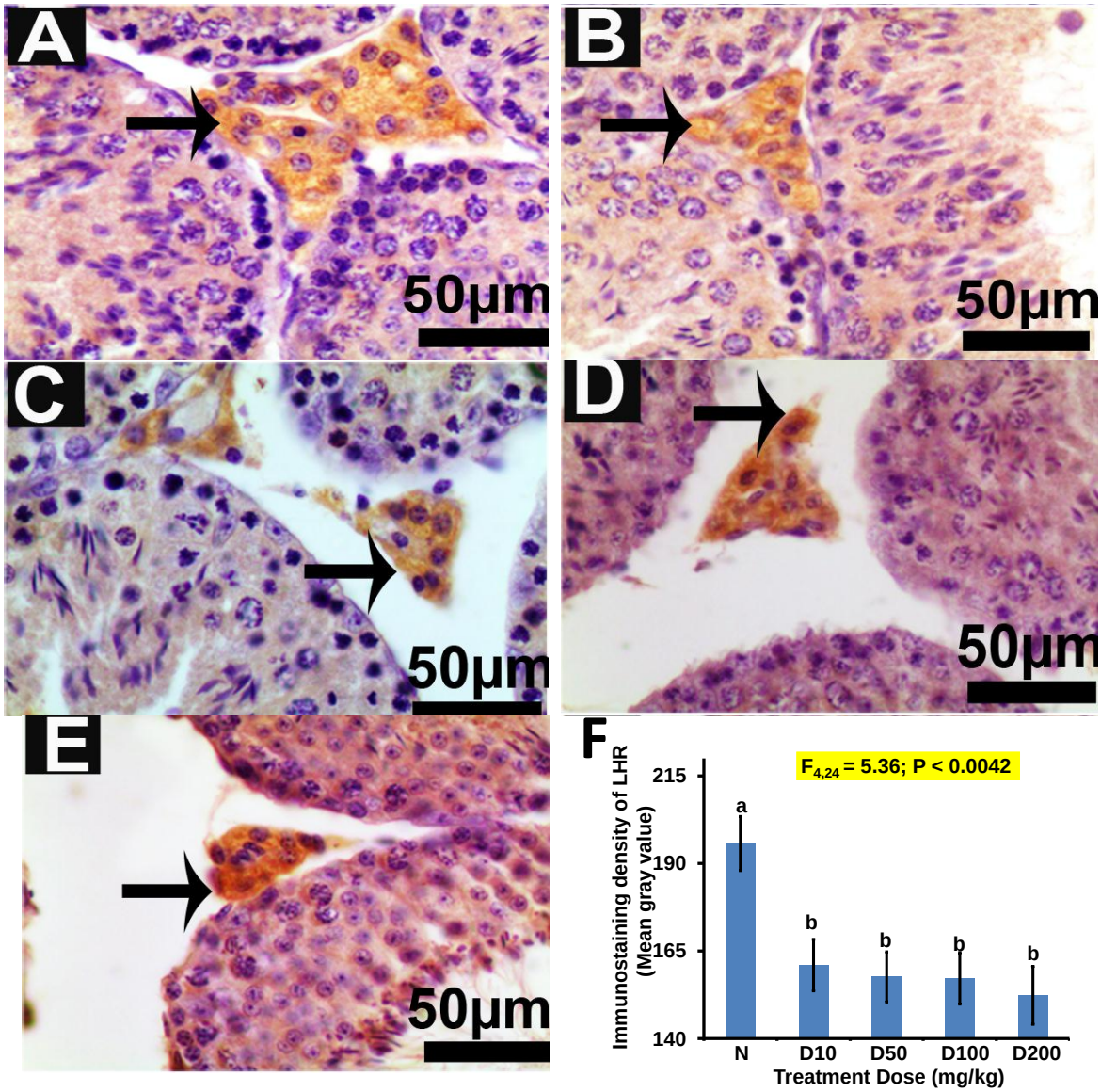


Fig. 24. Effect of diosgenin on LH receptor expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of LH receptor

[#]N- Control. *D – Diosgenin.

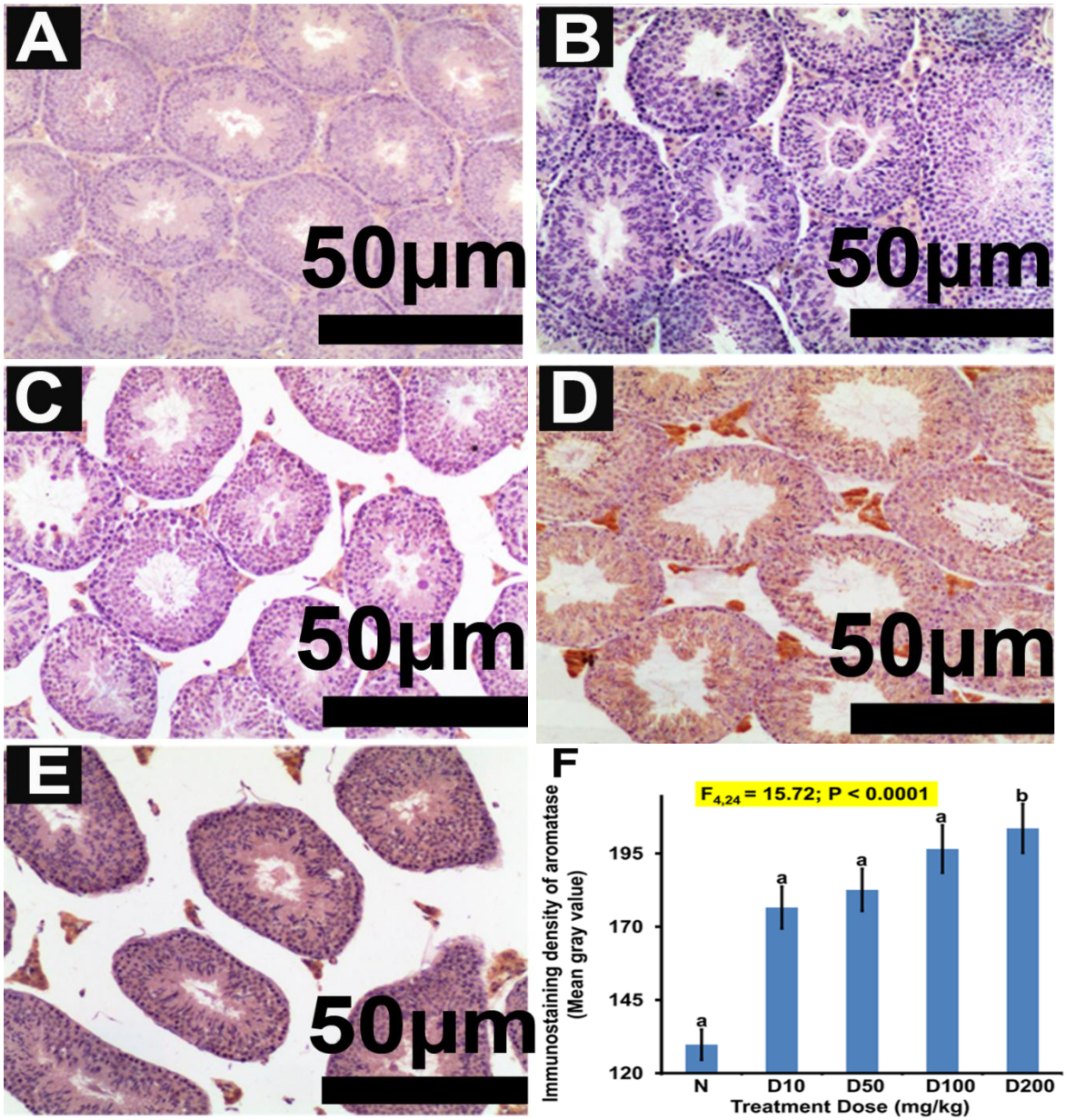


Fig. 25. Effect of diosgenin on aromatase expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of aromatase

#N- Control. *D – Diosgenin.

followed with impaired spermatogenesis. However, we observed that normal control group testis showed a light immune stained Leydig cells and some Sertoli cells in seminiferous tubule contributing to controlled aromatization of testosterone into estradiol. Herein, the immunostaining density of aromatase P450 (mean gray value) in normal control group was recorded as 129.73 ± 5.18 whereas in diosgenin treated group 176.55 ± 7.17, 182.57 ± 7.19, 196.52 ± 8.13 and 203.57 ± 8.38 in D10, D50, D100 and D200 respectively ($F_{4,24} = 15.72$; $P < 0.0001$).

Bcl-2 family genes are known for their anti-apoptosis or pro-apoptosis property and play an essential role in process of mitochondrial apoptosis pathway. This gene is also involved in regulation of spermatogenesis as dead cells or damaged spermatozoa needs to remove from seminiferous tubules. Many other factors such as environmental toxicants, many cytotoxic agents (pesticides), excessive heat and radiation from different sources may increase the process of germ cell apoptosis in mammalian testis. Bcl-2 expression may directly altered by exposure of any cytotoxic compound leading into abnormal spermatogenesis in male testis. In normal mice testis, its prime location is germ cell, spermatocytes (primary and secondary) and developing spermatozoa. Diosgenin, a potent steroid found in many leguminous seed increases the rate of apoptosis in testicular tissue of normal healthy mice possibly by up-regulating Bcl-2 expression. Immunohistochemical analysis of Bcl-2 protein revealed a significant Bcl-2 expression in testis of all diosgenin treated groups when compared to normal control group (**Fig. 26**). However, some Bcl-2 positive Leydig cells were also seen after diosgenin exposure. Herein, the immunostaining density of Bcl-2 (Mean gray value) in normal control group was

recorded as 84.39 ± 5.21 whereas in diosgenin treated groups 179.06 ± 7.13, 194.06 ± 9.1, 213.37 ± 10.22 and 229.04 ± 10.28 in D10, D50, D100 and D200 respectively ($F_{4,24} = 7.63$; $P < 0.0001$).

Caspase-3 belongs to cysteine proteases family and plays an essential role in process of apoptosis. It is well documented that abnormal formation of cell during spermatogenesis need to remove by a process of well-organized programmed cell death, apoptosis. Caspase-3 activation induces apoptosis by protein degradation within cell and stimulate Sertoli cell to phagocytes the cells. Moreover, abnormal activation of caspase-3 within cell may affect the process of sperm production as excessive death of germ cell affect fertility output. As a main and final perpetrator of apoptosis, caspase-3 activation may affected by environmental condition (heat, radiation and pesticides) and other cytotoxic agents stimulates the proteases to induce morphological and physiological changes leading into cell death. Diosgenin is widely used in pharmacology industry has been known for its protective effect against various kinds of diseases. However, in our study, it is concluded that diosgenin exposure in normal healthy mice induces testicular germ cell apoptosis through a series of events. Caspase-3 positive cells were observed mainly in germ cells and primary spermatocytes after diosgenin exposure. However, some spermatids and Leydig cell were also seen positive for caspase-3 protein expression. In our study, control group showed a light to moderate staining in germinal epithelial cells (spermatogenic cells series) and Leydig cells. Higher doses of diosgenin (D100 and D200) were more likely to affect germ cell survival and induce apoptosis of germinal cells and other cells produced during spermatogenesis. Leydig cells localized in

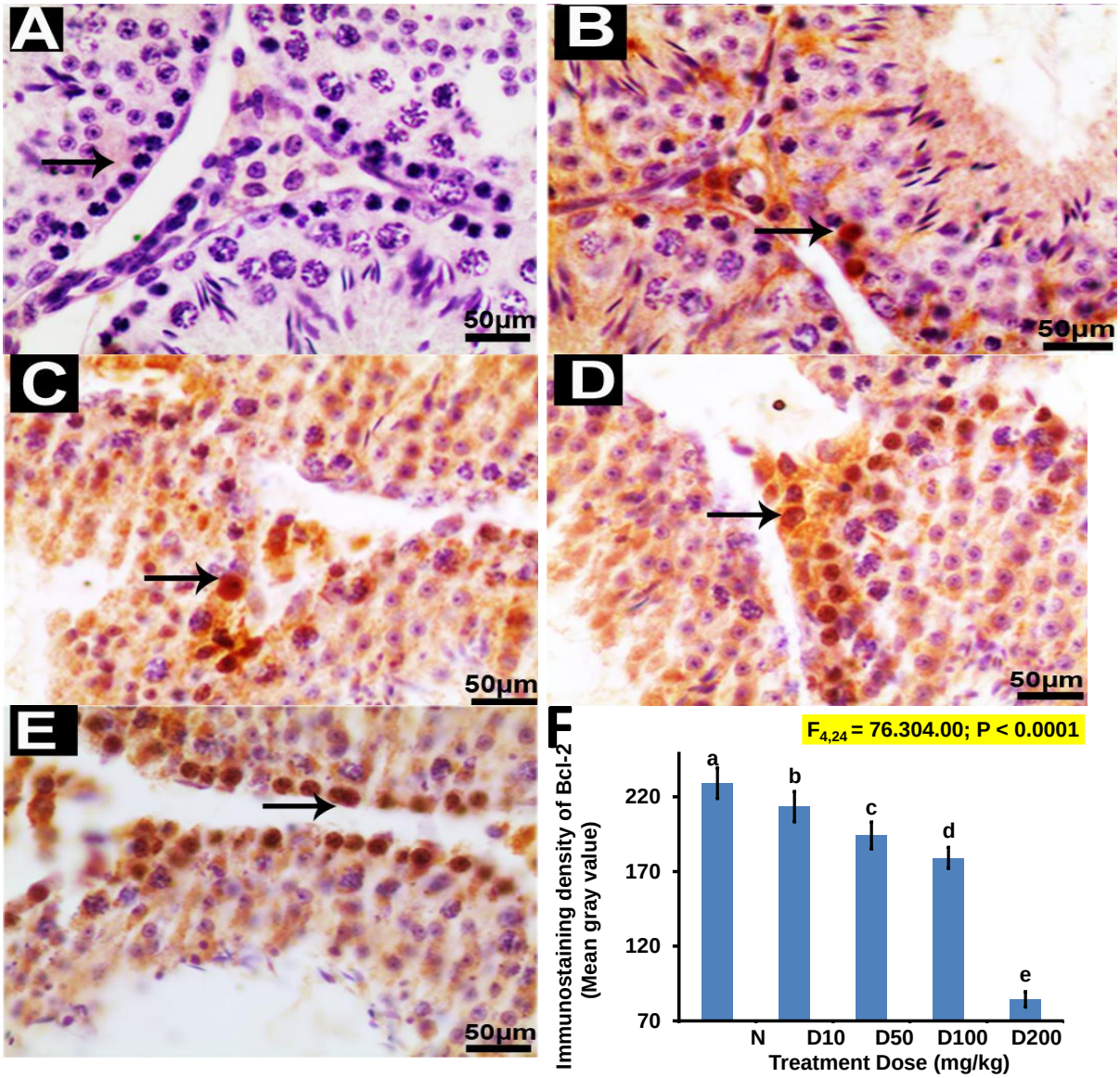


Fig. 26. Effect of diosgenin on Bcl-2 expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of Bcl-2

#N- Control. *D – Diosgenin.

interstitial compartment, responsible for production of testosterone were significantly affected by diosgenin administration leading into declined serum testosterone (**Fig. 27**). Herein, the immunostaining density of caspase-3 (mean gray value) in normal control group was recorded as 112.1 ± 4.09 whereas in diosgenin treated group 134.12 ± 6.05, 136.29 ± 6.24, 174.88 ± 8.29 and 193.0± 9.27 in D10, D50, D100 and D200 respectively ($F_{4,24}$ = 21.99; P < 0.0001).

So, taken all together, it seems diosgenin directly or indirectly interferes in the process of androgenesis and apoptosis by means of activation and suppression of several enzymes involved in testosterone synthesis and germ cell survival within testicular tissue. PCNA promotes epithelial germ cell proliferation in testicular compartment and its expression is sensitive with exogenous steroids. Diosgenin significantly reduced the PCNA expression in spermatogonia of testicular tissue. In this study, it has been observed that diosgenin administration affect the cholesterol transfer to outer membrane of mitochondria as there was reduced expression of StAR protein in testicular tissue. Other factor that strengthens our results is reduced expression of one of the steroidogenic enzyme, 3β-HSD, leading into decline in testosterone synthesis. LH receptors widely localized in interstitial compartment were also recognized with its low expression after diosgenin administration suggesting interference in gonadal-pituitary pathway. A balance level of estrogen is required for healthy spermatogenesis however; excess level of estradiol promotes fertility potential in male. Our data documented an elevated level of serum estradiol which is one of the causes of infertility. In addition to that, germ cell survival and apoptosis is modulated by several enzymes and necessary for maintenance of

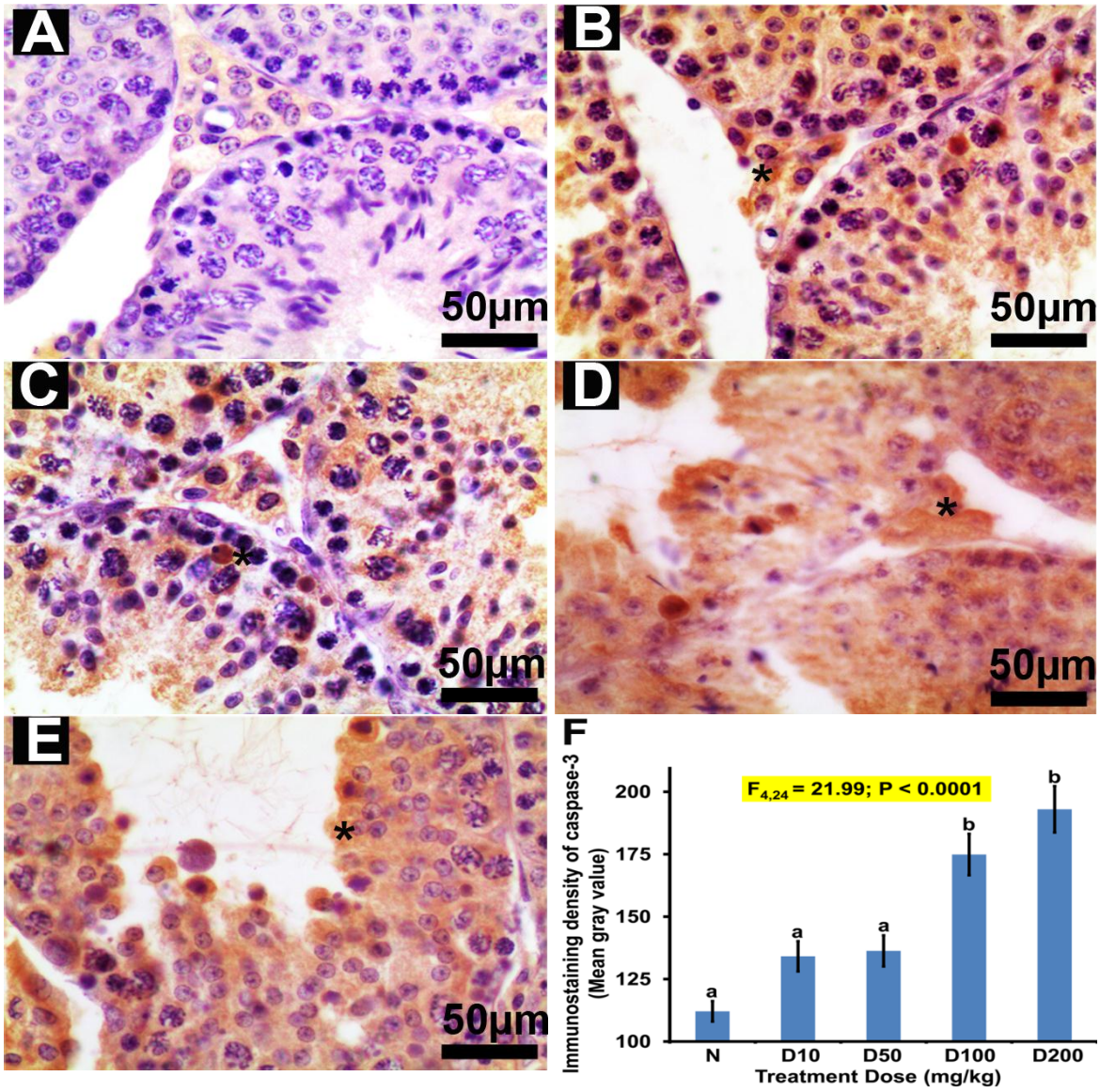


Fig. 27. Effect of diosgenin on caspase-3 expression in mice testis.

A-Normal control, **B**- diosgenin 10 mg, **C**-diosgenin 50 mg, **D**-diosgenin 100 mg, **E**-diosgenin 200 mg. and **F**-Immunostaining density of caspase-3

#N- Control. *D – Diosgenin.

homeostasis between productions of germ cell ratio with mature spermatozoa. However, some exogenous compound may increases the tendency of apoptosis in germ cells and other cells of seminiferous tubules causing sever testicular injury. In our research, we observed diosgenin exposure may directly or indirectly stimulated Bcl-2 and caspase-3 to encounter damaged cell. The chronic exposure of diosgenin altered several biochemical and physiological process of sperm formation and level of circulatory testosterone. Overall, low circulatory testosterone due to hazardous effect of diosgenin affects the spermatogenesis and finally fertility.

5.12. Diosgenin exposure declined the fertility potential rate

After successful insemination conformed by presence of sperm in female vaginal smear, pregnant female mice were carefully separated and observed. There was no post-implantation embryo loss recorded as absence of any vaginal bleeding in both diosgenin and normal control group. The chronic exposure of diosgenin significantly decreases the fertility rate in dose dependent manner when compared to normal control group (**Table 9**). At maximum doses of diosgenin (D100 and D200), it showed a decline in number of pregnant mice as compared to lower doses(D10 and D200). A normal control group showed a percentage of success pregnancy as 93.33 ± 1.12 whereas in diosgenin treated group it was recorded as 88.25 ± 1.45, 79.54 ± 1.85, 55.14 ± 2.01 and 32.36 ± 2.35 in D10, D50, D100 and D200 respectively ($F_{4,24}$ = 199.30; $P < 0.0001$). There were no noticeable changes in length and duration of

pregnancy throughout experiment. The average number of pups delivered by each female mice treated with different doses of diosgenin was significantly different among groups. The normal control group showed an ideal average number of litters delivered by each female which was 10.65 ± 0.45 whereas in diosgenin treated groups, it was recorded as 7.25 ± 0.52, 6.45 ± 0.61, 4.21 ± 0.27 and 3.21 ± 0.28 in D10, D50, D100 and D200 respectively ($F_{4,24}$ = 42.343; P < 0.0001). However, the weight of litters was not significantly affected in any doses of diosgenin when compared to normal control group. The normal group showed an average range of 1.90 ± 0.45 whereas in diosgenin treated groups it was recorded as 1.8 ± 0.36, 1.9 ± 0.48, 1.9 ± 0.51and 1.8 ± 0.21 in D10, D50, D100 and D200 respectively ($F_{4, 24}$ = 0.01731; P > 0.9994).

5.13. Statistical analysis

A statistical analysis of lipid peroxidation product and serum testosterone level with other parameters showed both positive and negative correlation and regression with respective parameters. **Fig. 28** clearly showed a noticeable negative correlation between testicular MDA as independent variable with serum testosterone level and sperm motility as dependent variable (**Fig. 28 A**, 22-A, r = 0.757; y = 8.379 - 0.778x and **Fig. 28 F**, 22-F, r = 0.776; y = 61.614 − 6.067x respectively). However, there was a strong positive correlation and regression observed between testicular

Table 9
Effect of diosgenin exposure on fertility potential of Swiss male albino mice.

Parameters	Experimental group					
	N	**D10**	**D50**	**D100**	**D200**	**F value**
Fertility Potential (%)	93.33 ± 1.12	88.25 ± 1.45	79.54 ± 1.85	55.14 ± 2.01	32.36 ± 2.35	199.30
Average Litter Size (number)	10.65 ± 0.45	7.25 ± 0.52	6.45 ± 0.61	4.21 ± 0.27	3.21 ± 0.28	42.343
Average Weight Of Litters (g)	1.9 ± 0.45	1.8 ± 0.36	1.9 ± 0.48	1.9 ± 0.51	1.8 ± 0.21	0.01731

Values are expressed as mean ± standard error mean (n = 5). One way ANOVA with Tukey post hoc multiple comparison test: Different letters within column signify differ significantly at $P < 0.05$ while same letter do not differ at $P < 0.05$. #N- Control. *D – Diosgenin.

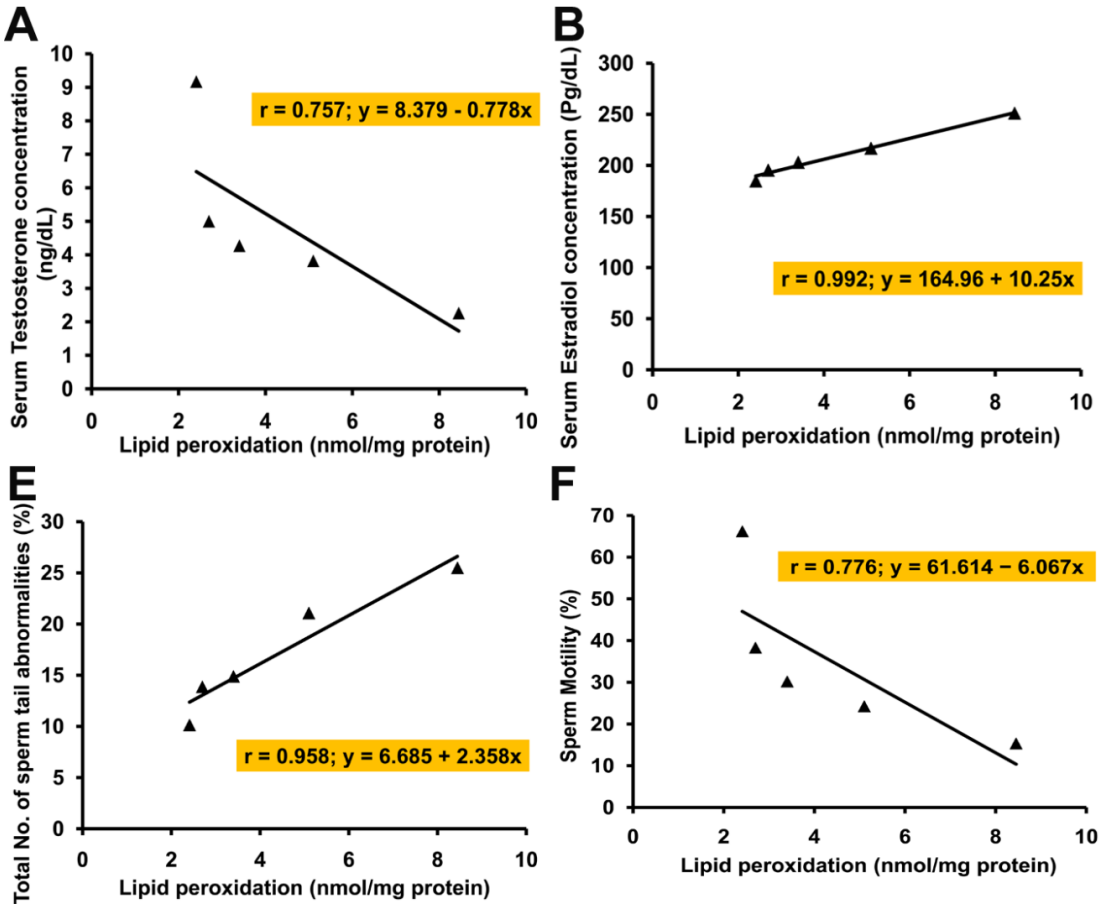


Fig. 28. Correlation and regression analysis between lipid peroxidation products with serum testosterone (A), serum estradiol (B), sperm morphological abnormities (E) and sperm motility (F) in normal and diosgenin treated mice.

MDA as independent variable with serum estradiol level and sperm abnormality as dependent variable (**Fig. 28 B**, 22-B, r = 0.992; y = 164.96 + 10.25x and **Fig. 28 E**, 22-E, r = 0.776; y = 61.614 − 6.067x respectively). A strong positive correlation and regression were observed between serum testosterone as independent variable with Johnson's testicular biopsy score (**Fig. 29 A**, 23-A, r = 0.890; y = 3.235 + 0.784x), mean seminiferous tubule diameter (**Fig. 29 B**, 23-B, r = 0.936; y = 131.144 + 16.121x), daily sperm production (**Fig. 29 C**, 23-C, r = 0.975; y = 3.436 + 0.768x) and cauda sperm concentration (**Fig. 29 D**, 23-D, r = 0.919; y = 12.742 + 2.088x) as dependent variable. The uniqueness and differences ($P < 0.05$) between groups such as cluster analysis (**Fig. 30**), principal component analysis (**Fig. 31**) and detruded correspondence analysis (**Fig. 32**) showed a significant difference among normal control group with all doses of diosgenin.

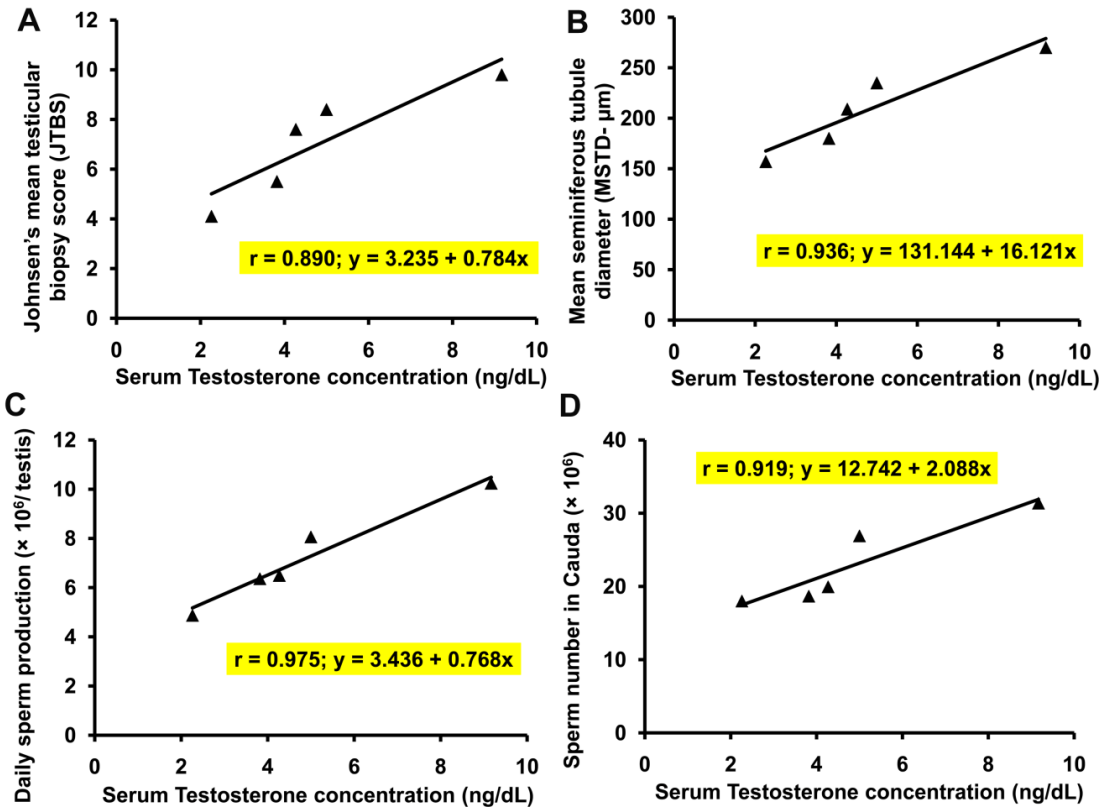


Fig. 29. Correlation and Regression analysis between Serum Testosterone concentrations with Johnson's mean testicular biopsy score (A), Mean Seminiferous Tubule Diameter (B), Daily sperm prodution (C) and cauda sperm concentration (D) in testis of normal and diosgenin treated mice.

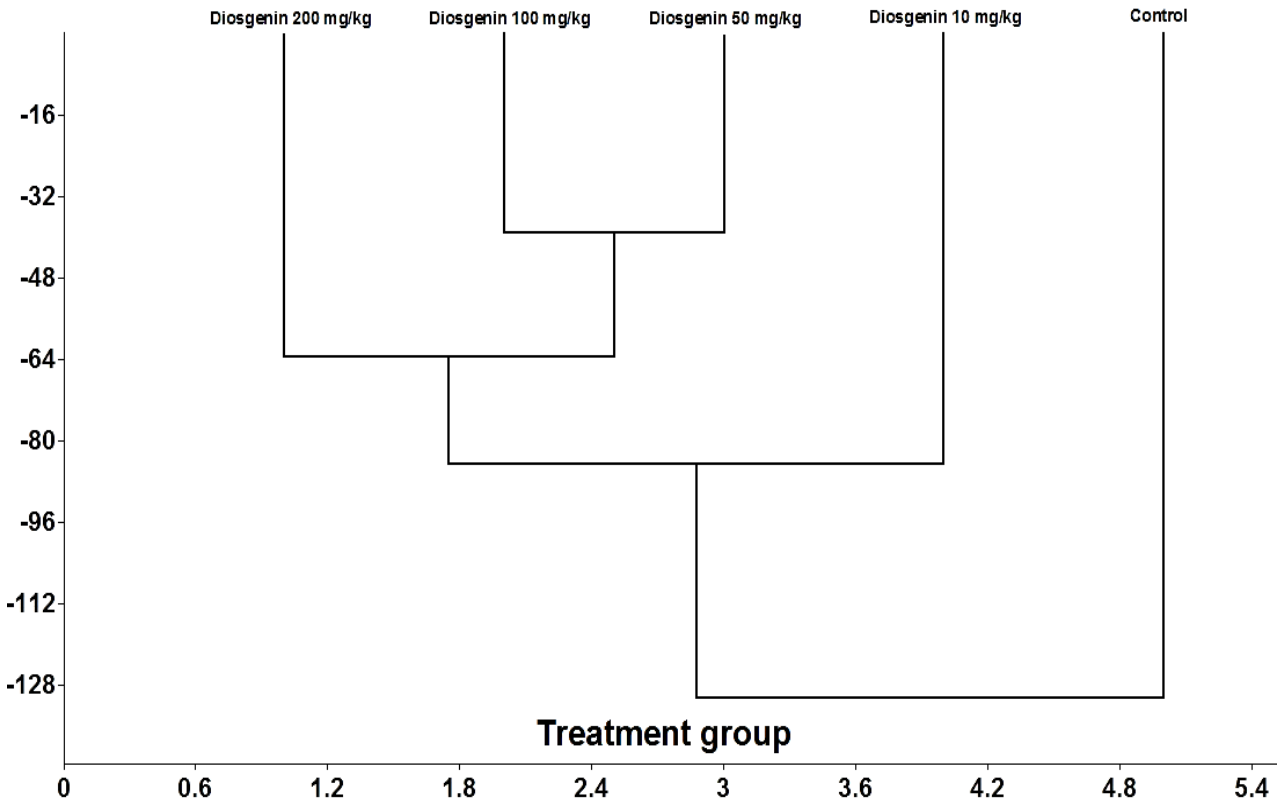


Fig. 30. Cluster analysis between diosgenin and normal control group.

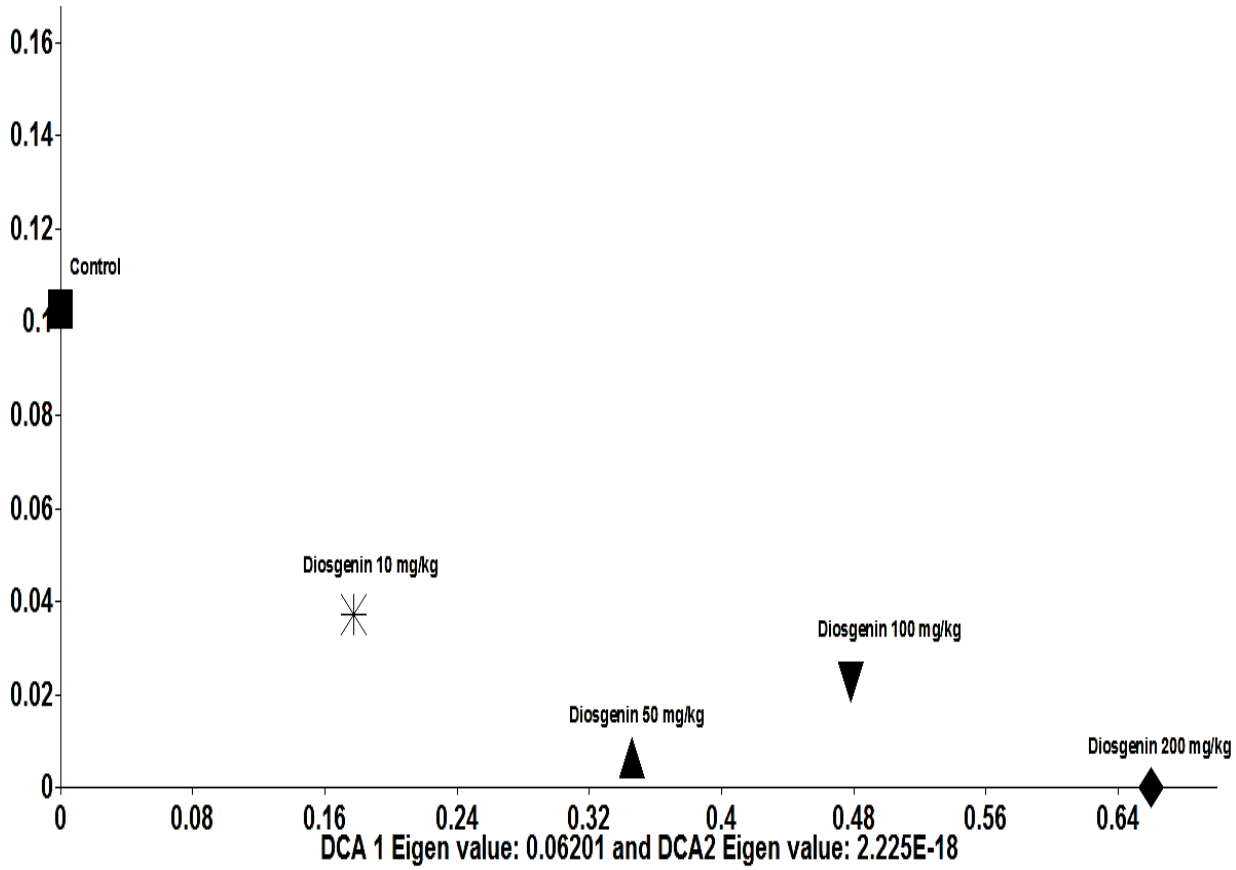


Fig. 31. Detrended correspondence analysis (DCA) between diosgenin and normal control group.

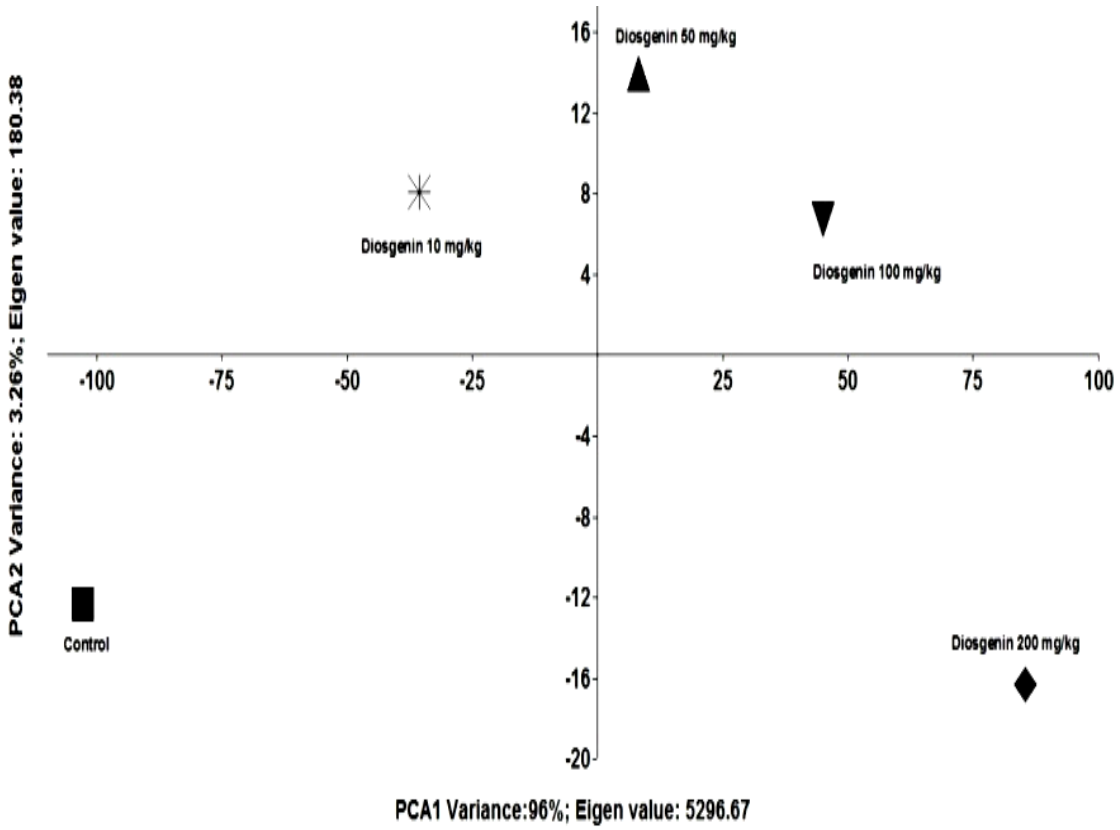


Fig. 32. Principal component analysis (PCA) between diosgenin and normal control group.

DISCUSSION

Diosgenin, a major steroid reported in fenugreek, *Costus* and *Dioscorea* species are known for its lowering blood sugar and lipid content in case of hyperglycemia (McAnuff*et al.,* 2005) and hyperlipidemia (Al-Matubsi*et al.,* 2011) respectively. Diosgenin has been widely used in industrial synthetic production of sex steroids like corticoids, progesterone, testosterone and estrogen (Chiang *et al.,* 2007). However due to its strong antioxidant effect against inflammations and free radical species, diosgenin has received attention towards its pharmacological effect on various kind of cancer and arthritis (Ma *et al.,* 2011). In an experimentally induced diabetic rat model, diosgenin enhanced glucose homeostasis in blood via improving hepatic enzyme function (McAnuff*et al.,* 2005b). Moreover, diosgenin has been used for estrogen deficiency and bone loss during female menopausal condition (Chiang *et al.,* 2011). Recently, diosgenin has reported as anti-depressive (Ho *et al,* 2012) and anti-ageing drug (Ho *et al*, 2012). However, here, completely lack of information about the role of diosgenin on reproductive function in vivo. Our findings claim that diosgenin act as testicular toxicants when it exposed in normal healthy mice. The present study highlighted the effect of diosgenin on sperm formation and testicular steroidogenesis. Although, estrogenic activity of diosgenin has been already reported by Aradhana *et al.* (1992) and Accatino *et al.*(1998) however, recently, report by Medigović *et al.* (2014), leading a lot of confusion and doubts about its effect on gonads and reproduction. According to their study, administration of diosgenin at doses of 20– 200 mg/kg body mass exposure in immature female rats did not significantly increases the wet uterine mass suggesting the other possible mechanism behind improved estradiol in menopausal condition. Still, there are rare or few clinical researches has been conducted on diosgenin effects on reproductive

parameters so most of the mechanisms by which diosgenin interferes in steroidogenesis as in case of menopausal condition is still unknown. Though, diosgenin is the main active constituent of fenugreek seeds and the extract of fenugreek seed have been shown to affect male and female reproductive functions. Till date, in our knowledge, this is first study on diosgenin action on normal male mice reproductive functions. To find out its interference in reproductive system, male reproductive parameters were analyzed and found a disturbed spermatogenesis and steroidogenesis.In this study, our finding concluded that administration of diosgenin causes testicular atrophy via degeneration of germinal epithelium along with other cell present in seminiferous tubule and decline in serum testosterone level. The germ cell loss triggering interrupted spermatogenesis and steroidogenesis affecting overall fertility potential.

Acute toxicity study of diosgenin revealed that it did not induce any noxious symptom in mice as there was no notable toxic effect on body physiology and well tolerated up to 600 mg/kg b.w. Previously, similar results have been mentioned by Qin *et al.* (2009) that diosgenin is well tolerated in rodents with oral doses of up to ~ 500 mg/kg. However, exposure of diosgenin with higher doses may interfere in reproductive function due to its estrogenic nature (Aradhana *et al.,* 1992; Accatino et al., 1998). In this research, we found a significant body and organ weight differences among groups. The higher doses of diosgenin (D100 and D200) were more likely to cause serious body weight loss comparison to lower doses of diosgenin (D10 and D50). The results support other findings that change in body weight may be influenced by diosgenin (Chen *et al.,* 2011).

The testis and other accessory sex organ weight were also significantly affected by chronic exposure of diosgenin suggesting its deleterious effect. The present study demonstrated that testis as well as accessory sex organs such as epididymis, seminal vesicle and prostate was showed a reduced weight. Previously, report by Kato *et al.* (2001), drug used for various pharmacological purpose (anticancer or anti-inflammatory) can induce depletion of testicular tissue resulting in declined testis weight. Moreover, diosgenin exposure at higher doses causes reduced weight of epididymis, seminal vesicle and prostate, due to imbalance of serum testosterone level. Although, reduction in testis and other accessory sex organs weights is androgen dependent, however till date there is no report on diosgenin interference in testosterone biosynthesis pathway. The weight of liver was much affected by diosgenin rather than kidney is cause of imbalance of hepatic function. There was a declined adrenal weight in diosgenin treated group which is an indication of a detrimental role on adrenal cortical zone. Previous report by Benghuzzi *et al.* (2003) explained reduction in adrenal tissue after diosgenin exposure to adult ovariectomized female rats suggesting possible endocrine complications specially defect in corticoids and sex hormones level. However, further research needs to require the actual mechanism behind diosgenin supplementation in normal and other model of reproductive parameters to compare its incorporation in steroidogenesis.

Progress of spermatogenesis and testosterone biosynthesis requires a number of key enzymes and exposure of exogenous steroid may interfere in their function. Several enzymes of liver and kidney are involved in drug metabolism and an

elevated level of serum hepatic and renal markers wasmostly known for damaged tissue. Diosgenin exposure for 40 days raised the serum ALP, SGPT, urea and creatinine level suggesting a deleterious effect on enzyme involved in drug metabolism. Moreover, the present study also demonstrated an elevated level of serum lipid biomarker such as cholesterol, triglycerides whereas HDL and LDH level were found to decline in their concentration.

Serum lactate dehydrogenase (LDH) is one of the key enzyme during spermatogenesis maintains continuous energy supply to developing spermatozoa and other cells of seminiferous tubules (Sinha *et al.,* 2001). Furthermore, the adequate amount of LDH is necessary for sperm motility however, excess level of serum LDH seems possible internal tissue injure. The LDH activity supposed to be affect by certain medicineand drugs. The present finding of our study clearly demonstrate the level of serum LDH was significantly higher in diosgenin treated group compared to normal control group suggesting a possible testicular damage in response to diosgenin exposure. In this concern, it is already well studies and reported by various researchers that therapeutic agent metabolism produces enormous ROS (Orisakwe *et al.,* 2003; Obianime *et al.,* 2011) thus affecting the enzyme functions (Rosenblum *et al.,* 1989). Subsequently, being a potent therapeutic drug in pharmacology, long term use of diosgenin may seriously affect the physiology of sperm production through alerting the testicular enzyme function necessary for spermatogenesis.

In male testes, seminiferous tubule is site for sperm production through a complex series of cell division of germinal epithelium into spermatozoa. Both spermatogenesis and steroidogenesis requires a lot of necessary testicular enzymes

and hormones to maintain continuous sperm equilibrium in adult male testis. Circulatory testosterones as well estradiol required in adequate amount for spermatozoa formation and maturation, impairments in circulatory testosterone: estradiol ratio leading into decline in male fertility potential (Carreau *et al.,* 2011; Rahman, 2008). Sperm motility, concentration and viability are usually most frequent parameter for accessing fertility potential. The sperm quality is possibly affected by a numbers of factors such as product of drug metabolite, excess generation of free radicals, temperature, age, hormonal imbalance, radiations and various diseases. The sperm motility is most susceptible parameter for fertility potential and regression may represents interrupted energy production due to lack of several essential enzyme required for glucose metabolism in testicular tissue. Other factors such as oxidative stress (Aitken *et al.,* 2011) may contribute spermatozoa immortality and viability since polyunsaturated fatty acid (PUFA) in plasma membrane of spermatozoa is easily peroxidised by free radicals (Zhou *et al.,* 2010). Diosgenin exposure adversely affected the all sperm parameters such as sperm motility, concentration, morphology and viability. Moreover, DNA damage in caudal sperm head was significantly higher in all diosgenin groups which could be a possible mechanism for diosgenin-related male reproductive toxicity or infertility. It is suggested that epididymal sperm head DNA damage was most likely affected with ROS due to sensitivity of nucleic acid to ROS. Another factor that may increase Sperm head DNA damage is impaired circulatory testosterone: estradiol ratio as spermatozoa needs adequate amount of testosterone and estradiol for spermiogenesis. Seminal fluid content also one of the essential factors responsible for sperm motion and viability (Cunha *et al.,* 1987) and decline level is associated with dysfunction of

sperm hence adversely affects the sperm parameters. Our finding clearly indicates that higher doses of diosgenin exposure interfere in sperm quality and long term use may influence on fertility potential.

Recently, phytosteroid also known as dietary estrogen has shown to interact and interfere with endocrine hormone function thus affecting the normal physiology and metabolism (Degen and Bolt, 2000). Steroid derived from plant source can mimics and interacts with estrogen receptor thus affecting the endogenous hormone level. In addition to that, an exogenous steroid may act as endocrine disruptor by modulating the endogenous sex hormone ratio (Safe, 2001). According to Alexander, (2014), phytoestrogen are known for adverse effect on reproductive physiology and affect production of sperm (Skakkebaek *et al.,* 2001). Many other environmental natural and synthetic substances such as xenobiotics and industrial wastes containing heavy metals, phenolic compound also interferes in endocrine function (Eddy *et al.,* 1996). Genestein, an isoflavones, well known for its antioxidant activity has now seriously major concerned about its estrogenic activity. Several metabolic enzymes involved in steroid production may easily altered by phytosteroid due to ability to mimic and bind with steroid receptors (Pihlajamaa *et al.,* 2011).

Previous report by Kuiper *et al.* (1998) claimed Genestein resembles structural similarity with estrogen and may compete towards higher affinity for its beta receptor. Testicular estradiol produced by aromatization of testosterone essential for sperm maturation and movement from lumen of seminiferous tubule towards epididymis however, an elevated level of both circulatory and testicular estradiol affects the affect sperm motility and final cause of poor fertility potential (Carreau *et*

al., 2011; Lubahn *et al.,* 1993). Another known estrogenic compound, Diethylstilbestrol (DES), reduces the percentage of epididymal sperm motility as well as concentration and weight of epididymis (Goyal *et al.,* 2001) in adult rat at a low dose of ≥8μg/day. It was suggested with above reference that even a low dose is enough to alter the epididymal function and thus detrimental to sperm activity. Previous report by Cornwall, (2009), already well reported that epididymal sperm motility significantly susceptible to androgen and hence essential for sperm maturation (Orgebin-Crist *et al.,* 1973). It is well known that amount of circulatory steroid directly influence the steroidogenesis in testis as well adrenal and excess or lack is cause of altered spermatogenesis, sperm maturation and motility. In addition to that, di-n-butyl phthalate (DBP) is a phthalate esters widely used as coating material for plastic items and can be easily released at high temperature (Mylchreest *et al.,* 2000; Thomas, 1984). Hence, being noticeable endocrine disruptive agents, DBP content in packed food and drinks can alter the endocrine function and interferes in reproductive process in gestational as well adult phase (Foster, 1998). Moreover, DBP administration at 0.5 to 500 mg/kg/day to pregnant rats caused complication of reproductive organ development and further fertility issue later in adulthood life (Zhang *et al.,* 2004). Furthermore, another study by Giribabu *et al.* (2012) explained about DBP interferes in steroidogenesis via down regulation of enzyme require for catalyzation of steroid into testosterone. Besides that, gestational exposure of DBP at 100 and 500 mg/kg/day significantly reduces the circulatory testosterone along with poor sperm quality. It was concluded with above reference that drug or any chemical may classified as estrogenic or anti-androgenic compound due to alteration in testosterone production either by imbalance of

testosterone: estradiol ratio or ability of binding with estrogen receptor (Ema, 2002). Another most possible way of low testosterone production is altering the essential enzyme involved in steroidogenesis (Giribabu *et al.,* 2012). Furthermore, there is a lot of evidence for resemblance of structural similarity of diosgenin with estrogen (Accatino *et al.,* 1998) and exclusively being used for commercial production of synthetic steroid hormone (Zenk, 1978).

In this study, we observed a noticeable low circulatory testosterone in all diosgenin treated mice in dose dependent manner. Diosgenin at 100mg/kg and 200mg/kg showed more adverse effect on testosterone concentration compared to lower doses of diosgenin. Subsequently, there was increased in plasma estradiol concentration in diosgenin treated groupsuggesting an excessive aromatization of testosterone into estrogen by aromatase (Brodie, 1985). Although, the reason behind the over production of estradiol beyond its normal range is largely unknown after diosgenin exposure. However, it is more likely due to structural similarity of diosgenin with estrogen hence may compete and binds with estrogen receptors in Leydig cells. Moreover, it is already well stabilized that presence of elevated level of circulatory or testicular estradiol has adverse effect on testicular as well epididymal sperm quality (Bolong *et al.,* 2009; Sumpter and Jobling, 2013). Furthermore, increased plasma estrogen level is associated with cancer of prostate, testis and breast tissue (Nelles *et al.,* 2011; Moore *et al.,* 2016) due to ability of estrogens to bind with its receptors. Another research report claimed drug with estrogenic activity mimic and binds with estrogen receptors (ER α and β) may act as carcinogens (Liang and Shang, 2013). However, role of estradiol in sperm maturation can't beignored due to

its requirement in some stages of spermatogenesis and maturation (Raymond *et al.*, 2010) hence plays a crucial role for testicular function. Moreover, presence of semen estradiol concentration is higher than female circulatory estradiol level suggesting a vital role for sperm motility, viability and concentration. Decline in semen plasma estradiol concentration is one of the most important factors involved in fertility potential of male individual.

As above discussed about estrogenic activity, diosgenin is already well reported as an estrogenic compound (Aradhana *et al.,* 1992). In this study, the decrease shown in the circulatory testosterone of male mice may be associated with the increased aggregation of free radicals are credited to the lethal or toxic effect of diosgenin as already reported with fenugreek extract (Ashban *et al.,* 2010; Al-Yahya, 2013). Testis is site of steroidogenesis, viewed as increasingly influenced to oxidative stress due to its high metabolic necessities and low testis weight resulted in poor spermatogenesis (Morales *et al.,* 2001). Diosgenin (steroid) being an active constituent of fenugreek seed so it might interfere in aromatization of testosterone into estradiol (E2) leads to expanded aromatase action, bringing about elevated levels of estradiol. Previous results by Kassem *et al.* (2006) demonstrated that fenugreek seed extract fundamentally diminished testosterone level in rabbits. According to him, diet containing 30% fenugreek seeds, altogether diminished testis weight and also male circulating testosterone.Similarly research done by Ibrahim and El-Tawill, (2010), fenugreek seeds powder (200 mg/rodent every day) for 30 days in male rodents have demonstrated reduced testicle weight alongwith huge decline in serum LH and testosterone hormones, indicated testicular toxicity. These findings showed,

a harmful effect of fenugreek seeds on Leydig cells function of seminiferous tubules which is bound to influence testis weight. These irregularity of reproductive hormone caused by disability of gonads and these weakened reproductive hormone system are almost certain bound to influence procedure of spermatogenesis.It indicatedcomprehensive impacts of plant source of diosgenin such as fenugreek, yam and crape zinger on male reproduction which may include oxidative stress in the gonads anddysfunction of reproductive hormone systemmay be connected with the generation of oxygen radicals. Another possibility of reproductive alteration may be change in male reproductive hormones that diminishes theproduction and quality of gametes (Tewari *et al.,* 1973; Mohan *et al.,* 2011; Ibrahim and El-Tawill. 2010; Singh *et al.,* 1972). In another hand, diosgenin has increases the adrenal cortical volume and density in ovx rat model suggesting its leading role in steroidogenesis (Ajdˇzanovi´c *et al.,* 2016). Besides that, other well known source of steroid diosgenin is reported in *C. specious* (Das *et al.,* 1970), extensively used for metabolic disorder and for anti cancerous activity. Although, the beneficial effect of *Costus* is well reported however lack of information on molecular mechanism of reproductive associated risk. Previous report by Tewari *et al.* (1973) revealed *C. speciosus* as anti-fertility plants due to its harmful effect on sperm production. According to him, diosgenin is potent antioxidant as similar to neoclinestrol (a well known estrogenic drug). In addition to that, estrogenic activity of saponin isolated from the rhizome of *C. speciosus* causes increased uterine weight with some morphological changes in uterine endometrial (Singh *et al.,* 1972). It has been also used for contraceptive purpose (Choudhury *et al.,* 2012). Several yam species of genus *Dioscorea,* known for raw material in pharmacological purpose due to its steroid compound, diosgenin

has been exclusively used worldwide. According to Sato *et al.* (2007), there are 9.5mg of diosgenin/1g of *Dioscorea*. Diet containing yam can significantly alter the fertility ability of adult due to its estrogenic nature well reported by various researchers (Das *et al.*, 2014). Another research report claimed for dose dependent effect on male fertility (Mohan *et al.*, 2011). He further explained that extract of *D. esculenta* significantly inhibited all sperm parameters along with marked decreased serum testosterone.

Previously, report of Chiang *et al.* (2011) suggested that diosgenin present in yam may protect ageing disorder due to its potent antioxidant activity against ROS. Another study by Higdon *et al.* (2001), claimed for its anti osteoporosis activity in menopausal ovx rat model. According to him, diosgenin supplementation during postmenopausal disordersmay improve strengthen of bone due to its structural similarity with estrogen. In this context, similar work has been conducted by Hung *et al.* (2014), who claimed diosgenin as good therapeutic drug for ageing disorder as there was increased in frame density and volume with decreased intensity of porosity in D- galactose-induced aging rat model. Most interestingly, there is another way of increasing the circulatory estradiol level via stimulating the adrenal cortical production of sex steroid with estrogenic activity of drug. Diosgenin supplementationinduces changes in adrenal gland suggesting a potential remedy for older aged people (Ajdˇzanovi´c *et al.* 2016). It seems from above studies that in case of aging disorder of male and female animal model, diosgenin exert estrogenic activity either by mimic like estrogen and its binding ability with estrogen receptors and increases the steroidogenesis. However, whether,diosgenin exposure in normal

healthy male mice will boost or diminish the circulatory testosterone level and its effect on spermatogenesis was still a matter for debates. In this study, we first evaluated diosgenin effect on normal male mice reproductive function and observed an alteration in steroidogenesis that significantly affected spermatogenesis.

As already well reported by several researchers that oxidative stress is process of naturally detoxify the ROS and other peroxides produced by aerobic metabolism of living being (Banerjee *et al.,* 2016). Normally, cellular antioxidant defense molecule scavenges the ROS and other peroxides produced by oxidative metabolism. Severe oxidative stress in tissue can affect the overall function via interruption in cell signaling and DNA function. Cellular antioxidant defense system such as SOD, GSH, catalase and GST scavenges the superoxide free radicals and other peroxide produced by excess oxidative stress thus detoxify the byproduct of lipid peroxidation.

However, due to exposure of various environmental factors and other metabolic disorder can influence the imbalance between oxidative stress and antioxidant defense species and can cause severe testicular damage (**Fig. 6** and **33**). There is many way of excess generation of ROS in testicular tissue. The most possible way that leading imbalance of MDA: antioxidant ratio is drug metabolism (Deavall *et al.,* 2012). Drug metabolism is complicated process in which

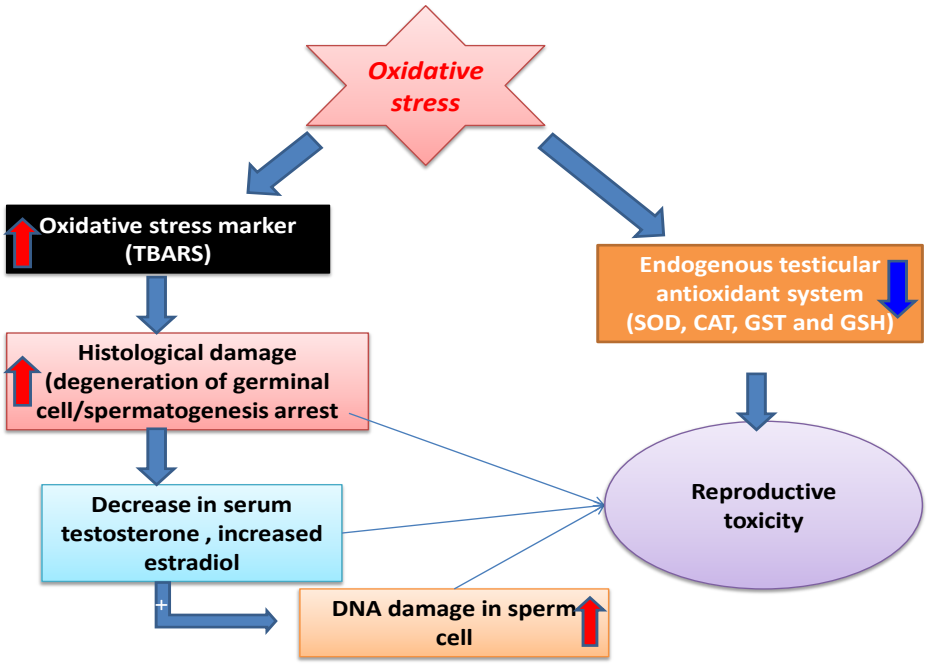


Fig. 33. Effect of oxidative stress and related reproductive toxicity.

biotransformation of drug generates reactive metabolites (Bhattacharyya *et al.*, 2014). These metabolites easily interact with nucleic acid and can alter the DNA activity thus exert an apoptotic role in cells (Sinha *et al.*, 2013; Liu *et al.*, 2002).

Study by Aitken and Shaun, (2008) reported exogenous steroid interferes in intra-testicular endogenous testosterone production in testicular tissue, and raises massive production of destructive ROS. Research by Forlenza and Miller (2006), explained abnormal increased oxidative stress in germinal epithelia may produce abnormal spermatozoa due to elevated level of testicular MDA content. Gonadotoxicity is also major concern with increased lipid peroxidation product as increased in concentration of free radical species adversely affect the rate of apoptosis (Agarwal *et al.*, 2008).

In male, testicular tissue continuously supplies the sperm cell through the process of spermatogenesis hence need of homeostasis between sperm production is necessary. Testicle tissue has ability to naturally maintain the normal and abnormal cells ratio of seminiferous tubule via increased free radical production. Some amount of free radicals is also produced by spermatozoa (Kanter *et al.*, 2011) however; excess production has been cause of male infertility (Sharma and Agrwal, 1998). Moreover, abnormal production of free radicals can facilitate the process of apoptosis in testicular germinal epithelium adversely affect the sperm production leading into decline in sperm mass. Additionally, it was suggested that impaired free radical species affects Leydig and Sertoli cell function, leading poor steroidogenesis along with reduced testicular weight (Morales and Heaton, 2001). The present study highlighted the effects of exogenous steroid (diosgenin) exposure on testicular

oxidative stress. In this study, we observed an elevated level of destructive MDA level in testicular tissue, may be due to cytotoxic nature of diosgenin on germinal epithelium cells and sperm cells. Here, therefore, most probably, decreased sperm mass, motility and viability observed in this study may have been caused by increased lipid peroxidation in testicular tissue and abnormal function of its accessory sex organs such as seminal vesicle. Our observations were similar with report of Dandekar *et al.* 2002 and Shewita *et al.* 2005. Despite the possible mechanism behind the raised oxidative stress level after diosgenin exposure in testis tissue, risk of an increase in infertility rat is issue. Similarly, altered testicular functionwith a known anti ulcer drug exposure was previously observed by Wang *et al.,* (1987) and Al-Nailey, (2010). According to them, use of higher doses of cimetidine drug for longer duration has been shown a deleterious effect on male reproductive system possibly by increase in oxidative stress level and generation of free radicals. Such therapeutic drugs, besides their clinical property may also exert another effect on fertility potential of individual (Wang *et al.,* 1987; Orisakwe *et al.,* 2003). Though, there is lack of information about the actual role of diosgenin on reproductive function at therapeutic dose levels however it is suggested that degeneration of testicular tissue may be due to cytotoxic nature of diosgenin that adversely elevated the testicular lipid peroxidation product.

Subsequently, generation of continuous ROS due to excess oxidative stress directly influence the ROS scavenger antioxidant enzyme functions. It is well known that oxidative stress as well antioxidant defense molecules are measure concern in development and maturation of sperm cells in testis and epididymis. Depletion of

endogenous antioxidant enzymes in testicular and epididymal tissue affect the sperm morphology and activity due to sensitivity of nucleic acid to ROS (Wang *et al.,* 2015; Sinha *et al.*, 2013). Earlier report by Al-Majed *et al.* (2006) has shown that abnormal morphology and change in DNA integrity of spermatozoa is one of the causes of decline in testicular glutathione level. Thus adequate amount of testicular enzymatic and non-enzymatic defense molecules required for homeostasis between oxidative stress and antioxidant activity.

According to Mishra and and Shaha, (2005), change in cellular glutathione content directly affect overall antioxidant defense and adversely affect the ratio of anti oxidant and cellular MDA concentration. Herein, we first time demonstrated that diosgenin inhibited antioxidant defense capacity in testis. In this study, our outcome showed that there is significant decrease in concentration of superoxide radical scavengers such as SODand GSH in testicular tissue. In addition to that, testicular concentration of Catalase and GST was also significantly reduced which is necessary for detoxification of freeradicals. Our study demonstrated that diosgenin exposure affected the testicular protein content may be due to drug metabolites, has been shown with noticeable inhibitory effect on protein biosynthesis in hepatic tissue. Similar report has been observed from Doreswamy *et al.* (2004). According to him, protein biosynthesis is susceptible to elevated MDA concentration due to adduct formation with nucleic acid. So, from above observation, it is suggested that drug used for antioxidant therapy for various purpose may exert another side effect to testicular tissue.

Several studies revealed germ cells dysfunction, disorientation of spermatids in lumen and spermatozoa dislocation, loss of Sertoli cell function is associated with reproductive toxicity and that were further involved in the decline of serum testosterone production (Richburg *et al.*, 1998). A normal testicular tissue consists of thousands of seminiferous tubule and surrounded by connective tissue. Each seminiferous tubule is independent of its on sperm production.Spermatogenesis is a very sensitive and complex process in which spermatozoa are produced from germinal epithelium through a series of meiotic and mitotic cell division. There are fourcategories of sperm cells (spermatogonia, spermatocytes, spermatids and spermatozoa) and nurse cells inside the lumen of seminiferous tubule while Leydig cells, known as steroidogenic site situated between interstitial spaces of tubules.Sertoli cells are randomly situated on basal lamina of seminiferous tubules and between the spermatogonia mother cells, well known for FSH receptor; provide necessary environment and nutrient to developing spermatozoa and other cells. Besides that, role of Sertoli cells in proliferation of germ cells is reported as very important.

In this study, mice treated with diosgenin showed a marked variation in ultra structure of testis when compared with normal control group. Our finding indicated that increased oxidative stress affected the testicular ultra structure due to increased rate of cell death trigger by ROS. Previous report also showed similar observation in oxidative stress mediated defect in testis. Another report by Nambu and Kumamoto, (1995), showed that Adriamycin (ADR), an anticancer drug, induces testicular disorganization and arrested the spermatogenesis. In addition to that, 17β-estradiol

exposure for one week induces detrimental effect to testicular tissue long with shrinkage of germ cell and spermatocytes with no sperm masses (Gill-Sharma *et al.,* 2001). Moreover, another research report by Takahashi and Oishi, (2003) revealed that there was negative impact on metabolic and progress of spermatogenesis after exposure of compound known for estrogenic activity. They further explained there was deterioration effect in germ cell and Sertoli cells resultininitiation of apoptosis. Most recently, Nandrolone decanoate (ND), a known synthetic anabolic steroid widely used as therapeutic purpose causes testicular atrophy, poor sperm production, degenerating effect on Leydig cells and germ cells, declined testosterone concentration and loss of fertility potential (Nagata *et al.,* 1995; Ahmed, 2015). Along with that, ND has shown an inhibitory effect on steroidogenic enzymes function (Koeva *et al.,* 2003).

Herein, we observed that diosgenin significantly affect the metabolic and physiological process in testicular tissue as there were noticeable degenerating cells in seminiferous tubule and seem an arrested spermatogenesis. Higher doses of diosgenin showed much damaging effect to germ cells and spermatocytes. However, normal control group showed a well organized tubule compartment. Each seminiferous tubule was oval or circular in shape and surrounded by connective tissues. There was adequate number of Leydig cells in interstitial area altogether with lymphocytes and blood vessels. In contrast, in diosgenin treated group, there were irregular shaped tubules with wide interstitial spaces, less or degenerating Sertoli cells between germ cells, splitting of meiotic spermatogonia from basal region, multinucleated giant cells, vacuolated spermatocytes, no or less mature spermatozoa

in lumen and few number of Leydig cells in interstitial spaces of seminiferous tubules. Furthermore, testicular atrophy and its related degenerating changes in testicular tissue has initiated after diosgenin exposure. The light photomicrographs of mice testes parenchyma in case of D10 mg/kg/body weight showed slightly shrinkage in tubule area, wider interstitial spaces, detached germinal cell from its basal germinal epithelial layer and distorted elongated spermatids. Histological section of mice treated with D50 mg/kg/body weight showed distinct narrow wider interstitial spaces along with irregular shrinkage of seminiferous tubules resulting in more interstitial spaces, some multinucleate giant cell and decline in Leydig cell and Sertoli cells number. The light photomicrographs of mice testes parenchyma treated with D100 mg/kg/body weight showed an arrested spermatogenesis. In this group, degenerating changes has been seen in basal germ cells and spermatocytes. Some faint color vacuolated giant cells noticed as proceeding for apoptosis. Leydig cells are irregularly located between the somniferous tubules. At highest doses of diosgenin (D200 mg/kg/body weight), complete mitotic arrest has been observed. There was significant sloughing of germ cells and spermatocytes, vacuolated multinucleated giant cell and necrotic cellular debris in lumen of seminiferous tubules. Altogether, it seems that higher dose of diosgenin significantly increases the germ cell loss followed with degenerating spermatocytes and spermatids. Our study is accordance with previous study of Al-Yahya, (2013); Kassem *et al.* (2006). According to them, testicular atrophy is due to bioactive steroid compound, diosgenin.

A significant reduction has been observed in epithelium diameters of seminiferous tubule in diosgenin treated group when compared to normal control group. This alteration in shape is possibly due to destructive effect of diosgenin causing shrinkage of seminiferous tubules, necrosis among germ cells and spermatocytes. Our data demonstrated for control group as 270 ± 5.25µm whereas in all diosgenin exposed group showed a declined diameter of 235 ± 7.35 µm, 209 ± 6.83 µm, 180 ± 5.47 µm and 157 ± 5.2 µm in D10, D50, D100 and D200 respectively. From above observation, it is suggested that degeneration and increased apoptosis in germinal epithelium influences the delaminating germ cells and other cells of spermatogenesis series. Another factor that can cause morphological changes of tubule is declined in testosterone level (Skakkebaek, 2001). Moreover, a trophy of seminiferous tubule also leading into declined in germ cell mass concentration thus affecting testicular sperm production.

On the other hand, to compare the maturity of seminiferous tubules, the mean Johnson's score was measured and found to be significantly affected. In control rats, a score of 9.8 ± 0.45 whereas, diosgenin treated group exhibited as 8.4 ± 0.40, 7.6± 0.36, 5.5 ± 0.41 and 4.1 ± 0.33 in D10, D50, D100 and D200 respectively. From above observation it seems that higher doses of diosgenin are more likely to cause testicular atrophy as well degenerating spermatogenic cells whereas normal control group showed a complete series of spermatogenic cells with different stages of spermatogenesis. It is suggested that higher doses of drug for long period increases the chances of testis toxicity.

In rodents and mammals, spermatogenesis can be easily suppressed by many known and unknown reason. Interruption in maturation of germ cell and spermatocytes significantly affect the overall sperm production. Our finding on total number of germ cells and Sertoli cells per tubule of testis reveals that there is significantly metaphase arrest have been seen in some spermatogonia, spermatocytes and spermatids. Other reports also confirm same observation on maturity failure of spermatogenic cells. In control group, seminiferous tubule was consisting of abundance of germ cells (60.5 ± 2.5), whereas, in diosgenin treated group it showed as 46.6 ± 1.8, 33.3 ± 2.1, 32.6 ± 2.6 and 22 ± 1.33 in D10, D50, D100 and D200 respectively. Form above observation, it is clear that spermatogonia, most sensitive and initiator of spermatogenesis, were mostly affected with higher doses of diosgenin. Furthermore, for maturation of sperm cells, Sertoli cells require to provide essential nutrition and estradiol thus decline number leading into altered spermatogenesis and maturation of sperm. In this study, we observed there was significant difference in number of Sertoli cells in diosgenin treated groups (10.9 ± 1.5, 5.6 ± 1.14, 3.8 ± 1.08 and 3.0 ± 1.0 in D10, D50, D100 and D200 respectively) when compared to control group (13.8 ± 1.1). In addition to that, declined in germ cell concentration further affected its mean proliferation rate and the number of spermatocytes (39.5 ± 3.03, 30 ± 2.12, 25.3 ± 2.15 and 23.4 ± 3.15 in D10, D50, D100 and D200 respectively) and spermatids (94.3 ± 1.7, 69 ± 2.1, 52.5 ± 2.8 and 42.2 ± 3.1 in D10, D50, D100 and D200 respectively). However, normal control group was observed with abundance of spermatocytes (47.2 ± 2.12) and spermatids (142.8 ± 2.5).

Glycogen content in testicular tissue is essential for continuous energy supply to spermatogenic cells in tubules. A decline in concentration of cellular glycogen content is may be due to high metabolic rate after drug exposure. In this study, PAS staining of testicular histology revealed that higher doses of diosgenin (D100 and D200) significantly affected the glycogen content in all cells present in spermatogenesis sequence. There was serious decrease in carbohydrate and fat ratio in basal germinal epithelium suggesting a poor blood supply towards tubules. The most affected cell was noticed as spermatogonia as there was serious reduction in carbohydrate and fat ratio in outer germ layer. Sertoli cells in higher doses were noticed with shrinkage cytoplasm thus affecting the balance between lipid molecules and glycogen amount. The reason behind the poor blood supply and decline in glycogen content either by increased oxidative stress or fibrosis on tunica albuginea is still remain unclear. However, it seems that decreased diameter of tubules, presence of extra tissue growth (fibroblast), uneven distribution of Leydig cells and blood vessels appeared to cause poor blood supply. Our observation on decline in sperm motility and viability are possibly due to alteration of oxidative phosphorylation after diosgenin exposure.

Androgen such as testosterone plays a major role to precede reproductive function in testis as well in its accessory sex organs. It also controls the synthesis and binding ability of receptors presents on testis, epididymis and seminal vesicle (French and Ritzen, 1973; Jones *et al.,* 1980; Higgins *et al.,* 1976). Previously, it is well reported by various researcher that sperm storage, nutrition and maturation are affected by exogenous steroid.

A detailed histological examination with Haematoxyline and eosin staining of caudal epididymis and seminal vesicle were done to observe a diosgenin related effect on testicular ultra structure. Herein, we observed that there were noticeable differences in ultra structure of caudal epididymis in diosgenin treated groups when compared to normal control group. A normal control group showed large round tubules with outer thin layer of columnar pseudo stratified epithelium and inside lined by stereocilia or microvilli. There was abundance of sperm mass in the lumen of caudal lumen. Whereas, there was decreased in size of tubules with disorganized columnar epithelium cell and noticeable loss in microvilli in diosgenin treated group. Sperm density was most affected with higher doses of diosgenin. In this study, we observed asignificant differences in ultra structure of seminal vesicle in diosgenin treated group when compared to normal control group.Seminal vesicle is an androgen dependant accessory sex organ of testis provides essential fluid consisting of fructose and prostaglandins to sperm (Cunha *et al.,* 1987). A thin layer of columnar pseudostratifiedepithelial cells with smooth muscles wall, villous mucosa and much finger-like projection along with many secretary glands towards lumen was observed in normal control seminal tissue whereas, in diosgenin treated groups, there was noticeable shrinkage in outer columnar cells along with decreased lumen area. It is suggested that diosgenin, an exogenous steroid, may imbalances in testosterone and estradiol ratio and possibly affected the changes in ultra structure of accessory sex organs (Bianco *et al.,* 2002). However, changes in ultra structure level of these organs could not be enough for explanation and needs further work on molecular level of steroidogenic protein expression.

Adrenal gland is second most steroidogenic site in mammals known for its vital role in physiology, metabolism and reproduction (Parker, 1995). Adrenal corticoids hormones responsible for glucose metabolism (glucocorticoids), minerals balance (mineralocorticoids) and production of testosterone, estrogen and progesterone (Raven and Hinson, 1996). Several endocrine disrupters are known for their inhibitory effect on cortical production of steroids (Mann, 1996; Harvey and Johnson, 2002) leading into sever metabolic disorder in body. Previous study on aquatic exposureof known estrogenic compound in fish inhibited the corticoids production (Hontela *et al.,* 1992). Furthermore, Harvey and Everett, (2003) explained that endocrine disrupters produce toxic effect via directly targeting adrenocortical steroidogenesis and produces adverse effect on endocrine physiology. In addition to that, another study revealed that administration of a synthetic androstane (cynotrimethyladrostenolone) in rat suppresses the steroidogenesis in adrenocortical tissue (Carthy *et al.,* 1996). Moreover, exposure of Letrozole (selective aromatase inhibitors) suppressed the adrenal production of estrogen, hence used to stop proliferation of breast cancer cells (Assikis and Buzdar, 2002). However, there is insufficient research on molecular and cellular mechanism behind the exogenous steroid exposure and suppression of adrenal cortical steroids. Diosgenin has known for effective role against menopausal condition hence being used as precursors for oral contraceptive and synthetic steroid hormones (HO *et al.,* 2012). Furthermore, report on menopausal rat model revealed decrease of cortical area in adrenal glands after diosgenin exposure. According to Benghuzzi *et al.* (2003), diosgenin significantly induces noticeable changes in adrenal cortex in ovx rat model of menopausal condition. Another report by Ajdˇzanovi´c *et al.* (2016) claimed changes

in volume density of cortical tissue after diosgenin exposure in an experimental rat model of the menopause. With contrast of above study, it seems structural similarity with sex hormone such as progesterone, estrogen and testosterone;exogenous diosgenin may affect overall steroidogenesis at testis as well adrenal level. Herein, we observed a decline in area of adrenocortical region with increased in medullar region after diosgenin treatment. In another hands, normal control group showed with a balance differentiation of cortical and medullar region. It is suggested that changes in steroidogenic tissue of adrenal due to increased oxidative stress leading a damaging effect.

PCNA (proliferating cell nuclear antigen), a 36-kDa nuclear protein, is used as potent biomarker for proliferating germ cells and spermatocytes in testicular tissue.Recently, there is trend to use PCNA protein for detecting proliferating rate of germ cells (Jaskulski *et al.,* 1998) after chemically induced testicular toxicity due to its DNA repair nature. PCNA also regulate cell cycle and its expression has been observed at G1 and S phase suggesting a crucial role in DNA replication.

Till now, there is no report on testicular expression of PCNA in mice. We first time evaluated diosgenin related effect on germ cell proliferation. Our study demonstrated that diosgenin exposure in normal healthy mice significantly affected proliferation rate of spermatogonia and spermatocytes because of decline in expression of PCNA protein in seminiferous tubules. In contrast with above text, normal control group showed adequate expression of PCNA in nuclei of outer germinal layer (spermatogonia). Moreover, some staining in spermatocyte (prophase I) was also noticed in their nuclear content however, other cells involved in

spermatogenesis like spermatids and spermatozoa were devoid of its expression. There were significant differences in immunostaining density of PCNA (mean gray value) among diosgenin treated groups and normal control group. In normal control group, density of PCNA was observed as 138.17 ± 8.66 whereas in diosgenin treated groups it was showed 138.86 ± 8.26, 131.11 ± 6.3, 112.66 ± 5.17 and 93.5 ± 4.13 in D10, D50, D100 and D200 respectively ($F_{4,\ 24} = 8.40$; $P < 0.0004$). It is suggested form above report on PCNA expression density that diosgenin significantly interfere in basal epithelium germ cell function as there was decline in PCNA expression in spermatogonia and spermatocytes. The mechanism behind the poor expression of PCNA may be due to elevated testicular MDA content or degeneration in germ cells. Some amount of expression has been seen in spermatogonia and spermatocytes in diosgenin treated group suggesting a DNA repair role of PCNA (Toschi and Bravo, 1988).

There are many enzymes involved in steroidogenic pathway that initiate and precedesteroidogenesis in gonads and adrenal tissue. Steroidogenic acute regulatory protein (StAR) transports cholesterol to inner mitochondrial membrane during steroidogenesis. Its expression in testicular tissue has been well reported in Leydig cells (LaVoie and King, 2009). Like other steroidogenic enzyme involved in steroidogenesis, StAR is also affected with exogenous steroids (Scott *et al.*, 2009). Hence, atrophy of testicular tissue adversely affects the Leydig cell number and function further affecting the intensity of StAR expression. In our study, we observed a declined in StAR expression. Our finding is accordance with recent study of Min and Lee, (2018). According to him, use of synthetic steroid, Nandrolone decanoate

(ND) even at low doses impaired steroidogenesis and suppress the testicular StAR expression. Another study revealed that there is decrease in testicular protein concentration of steroidogenic proteins after ND treatment (Barone *et al.,* 2017) suggesting a degenerating effect on Leydig cell. Though, since use of synthetic steroid have been exclusively used for therapeutic purpose however there is need of awareness on abuse of these drug due to risk of sever alteration in reproductive system that may cause sterility (De Souza and Hallak, 2011; Gold *et al.,* 2018). From above observation, it is clear that exogenous steroids directly target the Leydig cells in testicular tissue and adversely affect the steroidogenesis (**Fig. 34**).

Herein, we observed that there is significant reduction in StAR expression in diosgenin treated group when compared to normal control group. The immunohistochemical study of StAR in normal control group showed a positive expression in Leydig cells and in sperm massestowards lumen.The immunostaining density of StAR (mean gray value) in normal control group was showed as 196.37 ± 13.51 whereas in diosgenin treated group it showed as 183.92 ± 13.02, 142.87 ± 11.03, 112.21 ± 5.61 and 111.37 ± 5.58 in D10, D50, D100 and D200 respectively ($F_{4,\ 24} = 11.22$; $P < 0.0001$). From above observation, we concluded that higher doses of diosgenin were more seriously suppress the testicular StAR expression however; the lower doses were not much effective when compared to normal control group.

3β-hydroxysteroid dehydrogenase (3β-HSD) catalyzes the steroid into more potent form of sexhormones. In steroidogenesis, once StAR transported cholesterol to inner mitochondrial membrane, 3β-HSD become activated and catalyzes the steroid into progesterone, 17α-hydroxyprogesterone, androstenedione and

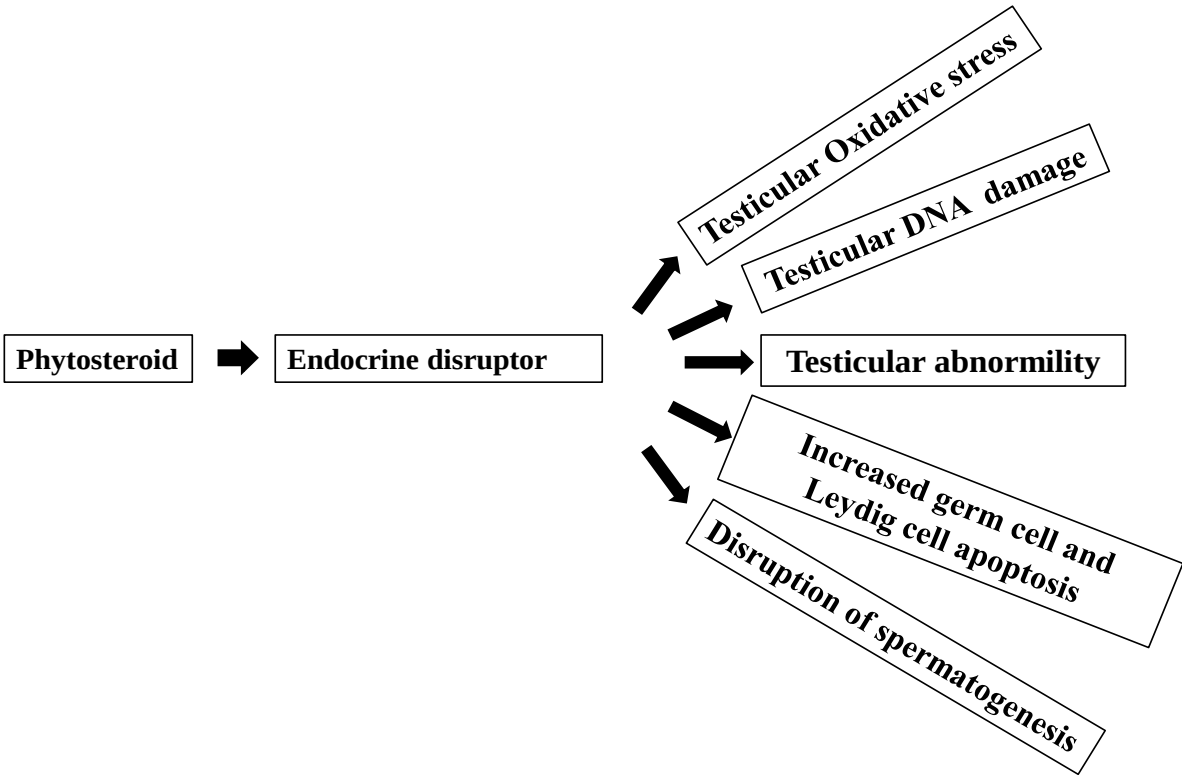


Fig. 34. Phytosteroid and testicular toxicity.

testosterone from pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA) and androstenediol respectively. Its primary location has been well documented in Leydig cells and sometimes in Sertoli cells (Pelletier *et al.,* 2001). Another hand, LH receptors (LHR) or human chorionic gonadotrophins (hCG) is well located in gonads (testis and ovary) as well in accessory sex organs (Tao *et al.,* 1995; Makino, 1973). LHR plays a major role in Leydig cells development and differentiation in the early phases of testicular cells development necessary for steroidogenesis (Ahmad *et al.,* 1973). It shows regulatory control on hypothalamic pituitary gonadal axis through high-affinity G protein coupled receptors (GPCRs). Moreover, in testis, cAMP independent messengers regulates activation of LHR, leading a controlled over steroidogenesis as well spermatogenesis in Leydig cells (Sharpe, 1987). In our study, we observed a decline expression of 3β-HSD and LHR in testicular tissue of diosgenin treated groups when compared to normal control group. Similar type of work has been conducted by Koeva *et al.* (2003); Ahmed, (2015); Barone *et al.* (2017). According to them, use of ND even a short period of time has shown to cause suppressed testicular steroidogenic enzyme activities (3β-HSD, 17βHSD, StAR and CYP17). Hence, it is suggested from above observation and previous study by various researchers that misuse of exogenous steroid/drug interferes in endocrine function as well as steroidogenic enzymes that leading into suppression of 3β-HSDand LHR expression.

The majority of 3β-HSD and LHR expression was documented in Leydig however other cells were devoid of expression. In higher doses of diosgenin, the immunostaining density of 3β-HSD (187.36 ± 6.79, 176.38 ± 6.24, 151.42 ± 5.22 and

142.12 ± 5.87 in D10, D50, D100 and D200 respectively, $F_{4, 24} = 13.19$; $P < 0.0001$) and LHR (160.98 ± 7.35, 157.61 ± 7.16, 157.2 ± 7.35 and 152.35 ± 7.72 in D10, D50, D100 and D200 respectively; $F_{4, 24} = 5.36$; $P < 0.0042$) were significantly reduced compared to lower doses of diosgenin. In normal control group, the immunostaining density of 3β-HSD and LHR was observed as 195.76 ± 7.4 and 195.68 ± 8.27 respectively. Herein, an interrupted steroidogenesis has been documented in this study after diosgenin treatment to healthy normal mice as there was decline in expression of 3β-HSD and LHR testicular tissue.

In testicular tissue, some amount of testosterone is metabolized into estradiol by cytochrome P450 aromatase, also known as CYP19 (Simpson *et al.,* 1994). The prime location of aromatase P450 enzyme is in Leydig cells and Sertoli cells of seminiferous tubules however some expression has been also reported in mouse germ cells (Nitta *et al.,* 1993). Furthermore, the expression of aromatase P450 is modulated by estrogen receptors via cell signaling. Previous report well documented that male testis needs both testosterone and estradiol for healthy spermatogenesis and lack of aromatase leading into interrupted spermatogenesis (Robertson *et al.,* 1999). However, there is adverse effect on reproductive function with over expression of aromatase gene has been well documented (Li *et al.,* 2001). Elevated level of 17β-estradiol has shown to cause testicular cancer or under active testicles (Li *et al.,* 2006; Gill-Sharma *et al.,* 2001), leading sterility in male individual. Furthermore, another study revealed that use of synthetic steroid altered the aromatase expression causes imbalance of testosterone and estradiol ratio (Barone *et al.,* 2017). Interestingly, a recent work of Cirelli *et al.* (2017) reported effect of microgravity on

aromatase expression in Sertoli cells. According to him, elevated level of testicular estradiol due to increased aromatase expression is cause of sterility and tumor in testis. Moreover, some known aromatase inhibitor such as letrozole has been exclusively used in case of over expression of CYP19 gene to suppress the aromatization of testosterone into estradiol (Schlegel, 2012). In addition to that aromatase is also sensitive to many exogenous steroid and environmental circumstances as altered aromatase activity contributed to the high estradiol, could cause serious fertility issue in male. Our study demonstrated that there was exclusively increased in aromatase expression in testicular Leydig cells after diosgenin treatment (176.55 ± 7.17, 182.57 ± 7.19, 196.52 ± 8.13 and 203.57 ± 8.38 in D10, D50, D100 and D200 respectively) when compared to normal control group (129.73 ± 5.18) (**Fig. 35**). However, in normal control group, it showed a light immune stained Leydig cells and some Sertoli cells in seminiferous tubule showed a controlled aromatization of testosterone into estradiol. From above observation, it is suggested that diosgenin may act as stimulator during testosterone metabolism into estradiol leading into up regulation of the aromatase expression.

In male testis, spermatogenesis is complex process, produces enormous amount of spermatozoa in lumen however due to various unknown factors some cells of spermatogenic series undergo morphological deformity. Thus, herein, a need of selective cell death in seminiferous tubule in order to maintain healthy sperm production (Aitken and Mark, 2012). Cell death is also known as apoptosis that initiate the degeneration of cytoplasmic, protein and nucleic acids content through the activation of various cleavage enzymes (Budihardjo et al., 1999; Elmore, 2007).

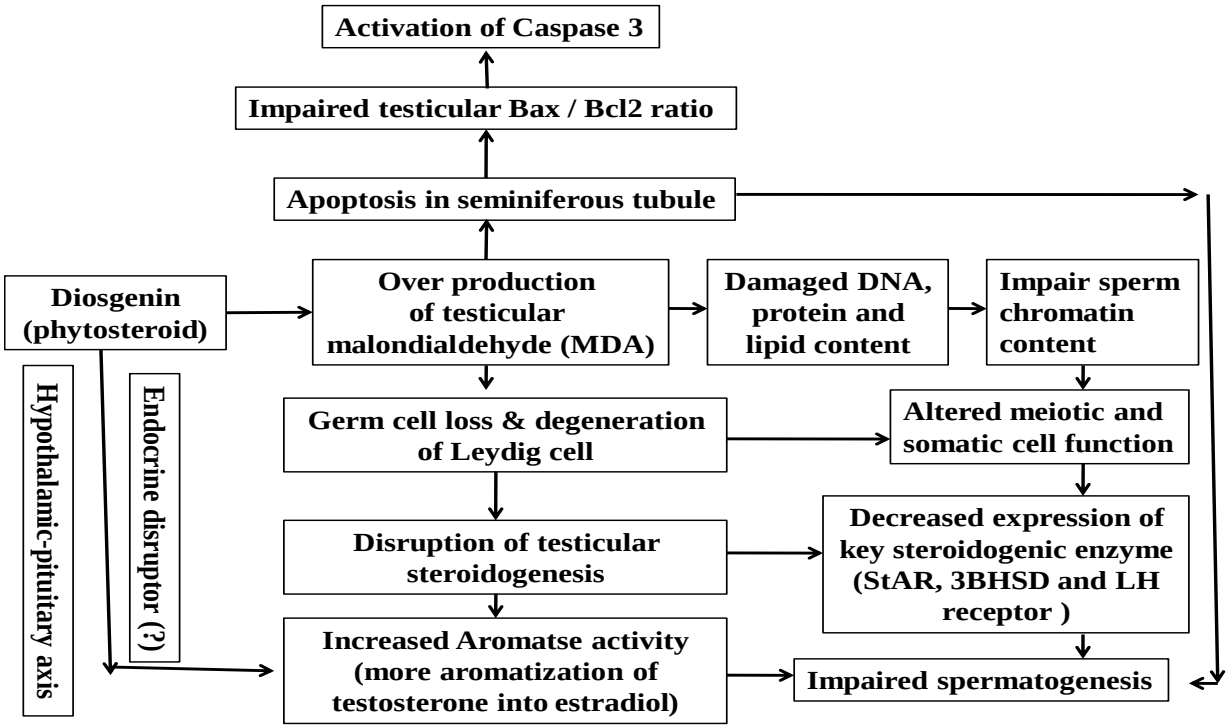


Fig. 35. Possible mechanisms of action of diosgenin on testicular toxicity.

Bcl-2 family genes are known for their anti-apoptosis or pro-apoptosis property and play an essential role in process of mitochondrial apoptosis pathway (Marsden *et al.,* 2002). Bcl-2 expression has been well documented in germ cell, spermatocytes (primary and secondary) and developing spermatozoa (Elmore 2007). This gene is also involved in regulation of spermatogenesis as dead cells or damaged spermatozoa affect the quality of sperm parameter (Richard *et al.,* 2008; Kierszenbaum, 2001). Thus, abnormal formation of cell during spermatogenesis need to remove by a process of well organized programmed cell death, apoptosis. In another hands, caspase-3 belongs to cysteine proteases family and plays an essential role in process of apoptosis. Like Bcl-2, caspase-3 expressions also have been reported in germ cells, spermatocytes and rarely in Sertoli cells (Elmore, 2007; Chowdhury, 2008). Caspase-3 activation induces apoptosis by protein degradation within cell and stimulate Sertoli cell to phagocytes the cells. Moreover, abnormal activation of caspase-3 within cell may affect the process of sperm production as excessive death of germ cell affect fertility output. Many environmental factors (excessive heat and radiation) and certain chemicals (pesticides or drugs) may increase the process of germ cell apoptosis in testicular tissue via altered Bcl-2 and caspase-3 expression that stimulates the proteases to induce morphological and physiological changes leading into cell death (McIlwain *et al.,* 2013). Moreover, Li *et al.* (2009) demonstrated a dose dependent effect of apoptosis induced by BPA, a known estrogenic compound widely used in food industries for packaging of food and juices. According to him, BPA exposure has increased apoptotic expression of genes and suppressed the steroidogenic enzymes function leading into altered spermatogenesis. In this study, we observed an increased apoptosis rate in tubules of

diosgenin treated groups when compared to normal control group. Herein, Bcl-2 and caspase-3 positive cells were significantly observed mainly in germ cells and primary spermatocytes in diosgenin treated groups. However, some spermatids and Leydig cell were also seen positive for both proteins. However, normal control group showed a light to moderate staining for Bcl-2 and caspase-3 in germinal epithelial cells as well in spermatocytes (84.39 ± 5.21and 112.1 ± 4.09 respectively) when compared to diosgenin treated groups. Whereas, diosgenin treated groups showed significant increase in Bcl-2 (179.06 ± 7.13, 194.06 ± 9.1, 213.37 ± 10.22 and 229.04 ± 10.28 in D10, D50, D100 and D200 respectively: $F_{4,24} = 7.63$; $P < 0.0001$) and caspase-3 (134.12 ± 6.05, 136.29 ± 6.24, 174.88 ± 8.29 and 193.0 ± 9.27 in D10, D50, D100 and D200 respectively $F_{4,\ 24} = 21.99$; $P < 0.0001$) protein expression in testicular tissue. Our observation is accordance with the work of Chandrasekaran *et al.* (2006) who revealed induction of apoptosis after exposure of mono-(2-ethylhexyl) phthalate. According to their study, maximum number of cell apoptosis was noticed in spermatocytes as well in spermatids. In addition to that, Billig *et al.* (1995) demonstrated apoptosis in primary spermatocytes after administration of gonadotropin-releasing hormone inhibitor. Nevertheless, the mechanism behind the exposure of diosgenin related selective manner of apoptosis in testicular tissue leading a disruption of testicular tissue has been not studied. However, it is suggested that being a steroid, diosgenin exposure may induces nuclear chromatin condensation leading into apoptosis in cells of seminiferous tubule.

Since, diosgenin has shown estrogenic activity in female especially at menopausal condition and being used as oral contraceptive (Aradhana *et al.,* 1992)

thus, there is urgent need to clarify about use of diosgenin and caution for safe doses.Some amount of estrogen is produced in Sertoli cell and germ cell of seminiferous tubules for maturation of spermatozoa (Hess, 2003). As earlier described, structure of diosgenin is similar to estrogen and it is already reported exogenous steroid interfere in testicular steroidogenesis. Due to its steroid nature, it may interfere in Leydig cell function, leading less testosterone production. However, still, it is matter of debate, whether, diosgenin have direct interfered in Leydig cell aromatase activity, as in our study, we found significant low serum testosterone but raised estradiol level, known for male infertility. Moreover, we also observed a decline in serum LH and FSH level, leading into insufficient stimulation to Leydig and Sertoli cells that further affect proliferation and maturation of cells in seminiferous tubules. This could explain possible mechanism behind impaired spermatogenesis leading significantly low serum testosterone levels in diosgenin treated mice were compared with control mice. However, more work required to find out the underlying mechanism behind diosgenin on aromatase activity to explain its role on steroidogenesis. Several steroidogenic factors such as StAR, LH receptor, P450 Aromatase and 3β-HSD expression at protein level as well gene level is needed to extract actual role of diosgenin on reproductive physiology. It is hypothesized that diosgenin exposure elevated oxidative stress that may interrupt the steroid enzyme function and various androgen receptor activity in testis of mice (**Fig. 33**). For these reasons, further studies are currently under way to explore the direct action of diosgenin on male reproductive functioning.

Fertilization is a very sensitive process which requires a specific hormonal environment in female reproductive tract to penetrate the zona pellucida of the egg (Aitken *et al.,* 1984). However, changes in sperm parameters such as motility and viability significantly affect the fertilization rate. Other factor like change in DNA integrity also affects the fertilization capacity of sperm. In testicular tissue, spermatozoa undergo some morphological changes through the process of spermiogenesis. Furthermore, another unique process happened when sperm enters in female reproductive tract, known as capacitation. Capacitation is series of biochemical transformations of sperm to make them enable for fertilization. Herein, sperm membrane undergoesmetabolic modificationsto make them hyper active such as an increase inphosphorylation rate, anionic changes and plasma membrane flexibility (Goodson *et al.,* 2011). A distinct asymmetrical flagella beating is seen during capacitation necessary for rapid movement to penetrate cumulus cells and zona pellucida (ZP) during fertilization (Jin *et al.*, 2007). Yanagimachi, (1994), explained that only capacitated sperms can bind to the ZP of the oocyte and undergo the acrosome reaction, a process that enables the sperm to penetrate and fertilize the oocyte. Hence, altered capacitation process reduces the chances of sperm to fertilize the egg leading into fertilization failure (Amieux and Mcknight, 2002).

In this study, we observed that chronic exposure of diosgenin significantly decreases the fertility rate in dose dependant manner. Diosgenin has decreases the percentage of fertility rateshown as 88.25 ± 1.45, 79.54 ± 1.85, 55.14 ± 2.01 and 32.36 ± 2.35 in D10, D50, D100 and D200 respectively ($F_{4,\ 24} = 199.30$; $P < 0.0001$) when compared to normal control group (93.33 ± 1.12). However, no noticeable

changes were found in length and duration of pregnancy. The average number of weight of litters was not significant in diosgenin groups (1.8 ± 0.36, 1.9 ± 0.48, 1.9 ± 0.51 and 1.8 ± 0.21 in D10, D50, D100 and D200 respectively: $F_{4, 24} = 0.01731$; $P > 0.9994$) as well control group (1.90 ± 0.45) suggesting no harmful effect on fetus. Moreover, our study demonstrated significantly decrease in litter's number in diosgenin treated group (7.25 ± 0.52, 6.45 ± 0.61, 4.21 ± 0.27 and 3.21 ± 0.28 in D10, D50, D100 and D200 respectively: $F_{4, 24} = 42.343$; $P < 0.0001$) suggesting a potent anti spermatogenic drug as loss of progressive movement and viability in sperm leading to a declined fertilization potential. However, normal control group showed as an excellent number of litter size which is recorded as 10.65 ± 0.45. From above observation it is clear that diosgenin exhibited as anti androgen and adversely affected the sperm quality and quantity. So from above observations and comparing with other research report it seems that chemical drug having various pharmacological properties and exclusively have been used as therapeutic agents may cause severe complication of reproductive systems leading into sterility. It is suggested from our study that in case of any disorder or oxidative stress diosgenin may act as potent antioxidant however its side effect can't be ignore due to its steroid nature. Not only use of drugs, even a daily dietary content of exogenous steroid may alter the endocrine as well paracrine systems.

SUMMARY

Summary

- Acute toxicity study of diosgenin revealed that a single dose of diosgenin at 100, 200, 400 and 600 mg/kg/b.w does not significantly alter the hepatic and renal functions.
- Diosgenin exposure elevated the hepatic and renal function and increases the disorder of lipid metabolism as there was increase in cholesterol and triglycerides level whereas serum HDL and LDH were significantly decreased.
- Diosgenin has been shown as anti-spermatogenic drugs/agents as there was decline in percentage of motility as well viability in mice.
- Daily sperm production and epididymal sperm concentration were also severely affected with diosgenin exposure.
- Diosgenin significantly increases the morphological abnormalities and sperm DNA damage possibly via increased oxidative stress or hormonal imbalance.
- An imbalance in serum LH, FSH, testosterone and estradiol significantly affected the proliferation and maturation of germ cell as well spermatozoa.
- Diosgenin induces oxidative stress mediated toxicity that influence abnormal physiological changes due to elevated MDA content has been observed in this study.
- Due to excess generation of ROS in testicular tissue, there is imbalance in ROS and antioxidant defense system leading into disrupted testicular function in diosgenin treated groups.

- Diosgenin significantly depleted the enzymatic and non enzymatic antioxidants status (SOD, GSH, GST and Catalase).
- Diosgenin increases the interstitial spaces between seminiferous tubule leading into uneven distribution of Leydig cells that adversely affect testosterone biosynthesis due to poor connectivity with blood vessels.
- Diosgenin exposure enhances the shrinkage of seminiferous tubule leading into a decreased in Johnson's score (assessment of spermatogenesis).
- Loss of weight as well pathological changes in ultra structure of cauda epididymis and seminal vesicle due to oxidative stress or reduced testosterone could be the one of reasons behind the abnormal sperms.
- Diosgenin not only disrupted testicular steroidogenesis, it also altered the adrenal cortiosteroidogenesis as there was decline in volume density of cortical area.
- Diosgenin administration has shown to suppress the cholesterol transport to inner mitochondria membrane as there was reduced expression of StAR protein in testicular tissue.
- Diosgenin, an exogenous steroid/drug interferes in endocrine function as well as steroidogenic enzymes that leading into suppression of 3β-HSD and LHR expression.
- Diosgenin has shown to upregulate the aromatase activity suggesting its interference in aromatase receptor.
- Diosgenin exposure induces the testicular atrophy leading into degeneration of testicular tissue and poor blood supply leading excessive cell death in tubule.

- Interrupted spermatogenesis due to diosgenin interference significantly increases the apoptotic rate via abnormal protein expressions of Bcl-2 and caspase-3 genes.
- Use of diosgenin for a longer duration can make sever fertility issue as herein we observed a decline in percentage of fertility potential as well decrease in litter numbers.

Limitation of the thesis and further approach to work

Based on the above observations and limitations of our thesis, we investigated diosgenin effect on hormonal, physiological, metabolic and some testicular protein expressions. Although, there has been a lot of experiments on animal model for therapeutic purposes, such as anti-diabetic, anti-inflammatory, anti-proliferative, anti-depressive and anti-aging etc, but its toxicity part is ignored. Previous and recent reports suggested that steroid may show harmful effect on reproductive function. Based on it, we are further interested to find out mechanism behind diosgenin related testicular toxicity at molecular as well as gene level. We also want to clarify whether change in exposure system (*in vitro* and *in vivo*) of diosgenin changes its efficacy or not. Maximum steroid treatment is done subcutaneously to limit its metabolism in digestive tract. Hence, change in route of exposure, diosgenin would be in direct contact of blood which may show some other changes in body. Furthermore, most interesting question which arises in our mind is that maternal exposure of diosgenin in gestational period would cause any reproductive alteration or related issue in offspring later in life or not.

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1. Presented a paper entitled "Effect of Diosgenin administration against anti-steroidogenic effect of copper sulphate and related testicular alteration in male mice" in an International Conference on "Biodiversity, Environment and Human Health: Innovations and Emerging Trends" organized by School of Life Sciences, Mizoram University, Aizawl and Association of Biotechnology and Pharmacy, India on 12-14 November, 2018.

2. Presented a paper entitled "Antifertility Screening of Fenugreek Containing Steroid Saponin" in a National Seminar on "Conservation and Sustainable Use of Medicinal and Aromatic Plants" organized by Department of Forestry, Mizoram University, Aizawl on 13-14 September, 2018.

3. Presented a paper entitled "Diosgenin affects Reproductive Dysfunction with Impaired Hormonal Imbalance and Oxidative Stress in Swiss Albino Mice" in a National Seminar on "Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India" organized by Department of Botany, Mizoram University, Aizawl on 30-31 March, 2017.

4. Presented a paper entitled "Effects of Tuibur (Tobacco Smoke Infused Water) on Spermatogenesis in Male Mice" in Mizoram Science Congress held at Mizoram University, Aizawl on 13-14 October, 2016.

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SEMINARS AND WORKSHOPS ATTENDED:

1. Participated in an International Workshop on "Snakebite Management" during 27th June – 03rd July, 2017 organized by Department of Zoology and Department of Biotechnology, Mizoram University, Aizawl.
2. Participated in a Workshop on "Capacity Building in Effective Management of Intellectual Property Rights (IPRs) in Biotechnology by Universities and Research Institutes in Mizoram" during 27th – 28th August, 2014 at Mizoram University, Aizawl and organized by Biotech Consortium India Limited (BCIL), New Delhi.

RESEARCH PUBLICATIONS:

1. **Maurya Khushboo[1]**, Meesala Krishna Murthy[1], Maibam Sunita Devi[1], Sanasam Sanjeev[1], Kalibulla Syed Ibrahim[2], Nachimuthu Senthil Kumar[2], Vikas Kumar Roy[1], Guruswami Gurusubramanian[1] (2018). Testicular toxicity and sperm quality following copper exposure in Wistar albino rats: ameliorative potentials of L-carnitine. *Environmental Science and Pollution Research*; 25 (**2**): 1837-1862. ISSN: 0944-1344. **Impact Factor: 2.8.**

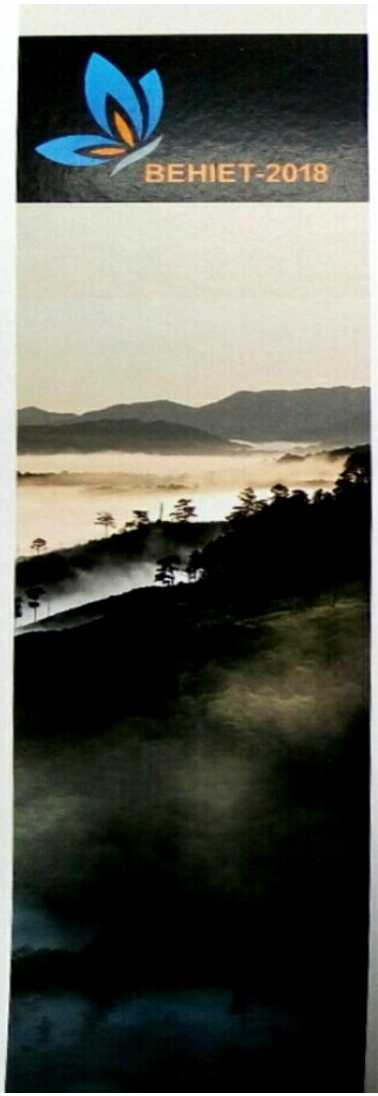
2. Sanasam Sanjeev[1], Meesala Krishna Murthy[1], Maibam Sunita Devi[1], **Maurya Khushboo[1]**, Zothanmawii Renthlei[1], Kalibulla Syed Ibrahim[2], Nachimuthu Senthil Kumar[2] , Vikas Kumar Roy[1] , Guruswami Gurusubramanian[1] (2019). Isolation, characterization, and therapeutic activity of bergenin from marlberry (Ardisia colorata Roxb.) leaf on diabetic testicular complications in Wistar albino rats. *Environmental Science and Pollution Research*; 26 (**7**): 7082-7101. ISSN: 0944-1344. **Impact Factor: 2.8.**

3. Bidanchi R Momin[1], Vikas Kumar Roy[2], Sanasam Sanjeev[3], **Khushboo Maurya**[4] and Guruswami Gurusubramanian[5] (2018). Cadmium Toxicity and Its Effect on Testicular Dysfunction in Male Rats. *Science and Technology Journal*; 6 (**1**): 62-67. ISSN: 2321-3388.

4. Vikas Kumar Roy [a], Vanlal Peki[a], Maibam Sunita Devi[a], Sanasam Sanjeev[a], **Maurya Khushboo[a]**, R. Zothansanga[b], Kalibulla Syed Ibrahim[b], Nachimuthu Senthil Kumar[b], Guruswami Gurusubramanian[a,*]. (2017). Biosterilant effects of Bacillus thuringiensis kurstaki HD-73 extract on male Wistar albino rats. *Theriogenology*; 88: 73-83. ISSN: 0093-691X. **Impact Factor: 2.136.**

5. Sanasam Sanjeev[1], Maibam Sunita Devi[2], **Khushboo Maurya[3]**, Vikas Kumar Roy[4] and Guruswami Gurusubramanian[5] (2017). Diosgenin: Therapeutic Action, Pharmacology and Applications. *Science and Technology Journal*; 5 (**1**): 7-15. ISSN: 2321-3388.

6. Maibam Sunita Devi[1], Sanasam Sanjeev[2], **Khushboo Maurya[3]**, Vikas Kumar Roy[4] and Guruswami Gurusubramanian[5] (2017). Implications of Use of Tobacco Smoke Infused Water. *Science and Technology Journal*; 5 (**1**): 22-31. ISSN: 2321-3388.



The 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) &

International Conference on
Biodiversity, Environment and Human Health: Innovations and Emerging Trends
(BEHIET 2018)

Organized by

School of Life Sciences,
Mizoram University,
Aizawl, Mizoram,
India

Association of Biotechnology
and Pharmacy (ABAP),
India

CERTIFICATE OF PARTICIPATION

This is to certify that ~~Prof.~~ / ~~Dr.~~ / Mr. / Ms. Khushboo Maurya
has ~~participated~~ / presented / oral / ~~poster~~ in the 12th Annual Convention of Association of Biotechnology and Pharmacy (ABAP) & International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends (BEHIET 2018) organized at the School of Life Sciences, Mizoram University, Aizawl, Mizoram 796 004 during November 12 to 14, 2018.

Title: Effect of diosgenin administration against anti-steroidogenic effect of copper sulphate and related testicular alteration in male mice.

Vice Chancellor,
Mizoram University

President,
ABAP

Dean, School of Life Sciences &
Organizing Secratrary

NATIONAL SEMINAR
ON
CONSERVATION AND SUSTAINABLE USE OF MEDICINAL AND AROMATIC PLANTS

CERTIFICATE

This is to certify that Prof./Dr./Mr./Mrs./Miss KHUSHBOO MAURYA **from** ZOOLOGY DEPARTMENT **has presented a poster/oral paper entitled** Antifertility Screening of Fenugreek containing Steroid Saponin (Diosgenin). **in the National Seminar on *"Conservation and Sustainable Use of Medicinal and Aromatic Plants"* held on 13th and 14th September, 2018 in the Department of Forestry, Mizoram University, Aizawl, Mizoram.**

Vice Chancellor
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Vice Chancellor
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CERTIFICATE

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(Dr. R. LALFAKZUALA)
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(Dr. LALDINPUIA)
Jt. Secretary

(Dr. R.K. LALLIANTHANGA)
Chairman

MIZORAM SCIENCE CONGRESS 2016 ORGANISING COMMITTEE

Sponsored by: NEC, DST (SERB) & MISTIC

MIZORAM UNIVERSITY

NATIONAL CONFERENCE ON

Impact of Climate Change on Biodiversity: Applications of Recent Technologies for Conservation of Threatened Species

22-24 September, 2016

Organised by : Department of Zoology, Mizoram University, Aizawl, Mizoram

Certificate of Participation

It is to certify that

Prof./Dr./Mr./Miss/Mrs. Khushboo Maurya, Mizoram University.

has participated in National Conference organized by Deptt. of Zoology, Mizoram University and presented in his/her key note / plenary lecture / technical paper / poster

on Diosgenin affects spermatogenesis in Swiss albino mice

He/She chaired a technical session.

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(Prof. R. LALTHANTLUANGA)
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(Prof. G.S. SOLANKI)
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STATE BIOTECH HUB (STB-HUB)
Mizoram University
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Certificate

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This certificate is presented to

Prof./Dr./Mr./Ms. Khushboo Maurya

for active participation in the

"Workshop on Capacity Building in Effective Management of Intellectual Property Rights (IPRs) in Biotechnology by Universities and Research Institutes in Mizoram "

at Mizoram University, Aizawl
from August 27-28, 2014

Sponsored by:
Department of Biotechnology, Government of India

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Environ Sci Pollut Res
https://doi.org/10.1007/s11356-017-0624-8

RESEARCH ARTICLE

Testicular toxicity and sperm quality following copper exposure in Wistar albino rats: ameliorative potentials of L-carnitine

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Received: 18 April 2017 / Accepted: 26 October 2017

Abstract Copper is a persistent toxic and bio-accumulative heavy metal of global concern. Continuous exposure of copper compounds of different origin is the most common form of copper poisoning and in turn adversely altering testis morphology and function and affecting sperm quality. L-carnitine has a vital role in the spermatogenesis, physiology of sperm, sperm production and quality. This study was designed to examine whether the detrimental effects of long-term copper consumption on sperm quality and testis function of Wistar albino rat could be prevented by L-carnitine therapy. The parameters included were sperm quality (concentration, viability, motility, and morphology), histopathology, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum urea, serum creatinine, serum testosterone and testis antioxidant enzyme levels (superoxide dismutase and glutathione-S-transferase), and biomarkers of oxidative stress (lipid peroxidation and expression of heat shock protein 70 in testis). Three-month-old male Wistar rats (n = 30) were divided into six groups as group 1 (G1, 0.9% saline control), group 2 (G2, CuSO4 200 mg/kg dissolved in 0.9% saline water), groups 3 and 4 (G3 and G4, L-carnitine 50 and 100 mg/kg dissolved in 0.9% saline water, respectively), and groups 5 and 6 (G5 and G6, $CuSO_4$ 200 mg/kg plus L-carnitine, 50 and 100 mg/kg dissolved in 0.9% saline water, respectively). Doses of copper (200 mg/kg) and L-carnitine (50 and 100 mg/kg) alone and in combinations along with untreated control were administered orally for 30 days. The following morphological, physiological, and biochemical alterations were observed due to chronic exposure of copper (200 mg/kg) to rats in comparison with the untreated control: (1) generation of oxidative stress through rise in testis lipid peroxidation (12.21 vs 3.5 nmol MDA equivalents/mg protein) and upregulation of heat shock protein (overexpression of HSP70 in testis), (2) liver and kidney dysfunction [elevation in serum ALT (81.65 vs 48.08 IU/L), AST (156.82 vs 88.25 IU/L), ALP (230.54 vs 148.16 IU/L), urea (12.65 vs 7.45 mmol/L), and creatinine (80.61 vs 48.25 μmol/

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-017-0624-8) contains supplementary material, which is available to authorized users.

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Published online: 04 November 2017

L) levels], (3) significant decrease in body (99.64 vs 106.09 g) and organ weights (liver—3.48 vs 4.99 g; kidney—429.29 vs 474.78 mg; testes—0.58 vs 0.96 g), (4) imbalance in hormonal and antioxidant enzyme concentrations [significant decline in serum testosterone (0.778 vs 3.226 ng/mL), superoxide dismutase (3.07 vs 8.55 μmol/mg protein), and glutathione-S-transferase (59.28 vs 115.58 nmol/mg protein) levels], (5) severe alterations in the testis histomorphology [sloughed cells (90.65%, score 4 vs 15.65%, score 1), vacuolization (85.95%, score 4 vs 11.45%, score 1), cellular debris along with degenerative characteristics, accentuated germ cell depletion in the seminiferous epithelium, severe damage of spermatogonia and Sertoli cells (73.56%, score 3 vs 0%, score 1)], (6) suppression of spermatogenic process [hypospermatogenesis (low Jhonsen testicular biopsy score 4 vs 9.5), decrease in tubules size (283.75 vs 321.25 μm in diameter), and no. of germ cells (81.8 vs 148.7/100 tubules), Leydig cells (5.2 vs 36.65/100 tubules), and Sertoli cells (8.1 vs 13.5/100 tubules)], (7) sperm transit time was shorter in caput and cauda and ensued in incomplete spermatogenic process and formation of immature sperm leading to infertility, (8) sperm quality was affected significantly [decreased daily sperm production (13.21 vs 26.9 × 10^6 sperms/mL), sperm count (96.12 vs 154.25 × 10^6/g), sperm viability (26.88 vs 91.65%), and sperm motility (38.48 vs 64.36%)], and (9) increase of head (32.82 vs 2.01%) and tail (14.85 vs 0.14%) morphologic abnormalities and DNA fragmentation index (88.37 vs 11.11%). Oxidative stress and their related events appear to be a potential mechanism involved in copper testicular toxicity and L-carnitine supplementation significantly modulated the possible adverse effects of copper on seminiferous tubules damage, testes function, spermatogenesis, and sperm quality. It was validated that the use of L-carnitine at doses of 50 and 100 mg/kg protects against copper-induced testicular tissue damage and acts as a therapeutic agent for copper heavy metal toxicity.

Keywords Copper toxicity · L-carnitine supplementation · Therapeutic agent · Sperm quality · Spermatogenesis · HSP70 expression

Introduction

Heavy metals are the major environmental contaminants of food chain not only negatively influences soil, air, and water but also become a considerable human, animal, and wild life threat due to its widespread use, persistence in nature, and potential toxic effects on human and animal health. Copper is one of heavy metals which are essential for utilization of iron, elastin and collagen synthesis, melanin production, integrity of the central nervous system, activation of numerous metalloprotein enzymes and non-enzymes, antioxidant defense, neuropeptide synthesis, and immune function (Harrison et al. 2000; Gaetke and Chow 2003; Uriu-Adams and Keen 2005; Bost et al. 2016). Nevertheless, this is a notable eco-environmental heavy metal contaminant due to its extensive usage in many enterprises like manufacturing of pesticides, leathers, electronic equipments, building and construction supplies, electric welding, water pipes, wood preservatives, transportation materials, and intrauterine contraceptive devices that causes undesirable effects including copper toxicity to the environment as well as human health (Saravu et al. 2007; Roychoudhury et al. 2016). The common route of toxic exposure of copper to humans and animals is in the form of copper sulfate through skin, eye, and inhalation. Copper content in food items, beverages, and drinking water is found to be above permissible levels and has been toxic when ingested at a dose of > 2 mg/kg. Chronic copper exposure is increasingly recognized as a public health issue; its early effects remain largely unknown. Ingestion of significant quantities of copper carries a risk of multi-organ failure and death. Copper sulfate ($CuSO_4$) is a strong oxidizing agent, passively diffuses into mucous membranes of small intestine, binds to cells during high load, and causing corrosive damage. Hemolytic anemia, oliguria, anuria, methemoglobinemia, liver failure, jaundice, coma, and convulsions are the clinical manifestations of copper toxicity (Sokol et al. 1990; Babaei and Abshenas 2013). Initially, copper accumulates in the liver and disrupts the liver's ability to detoxify elevated copper concentration in the body, thus it adversely affects the nervous system, reproductive system, adrenal function, connective tissue, and learning ability (Babaei et al. 2012). Liver and kidney tissue damage are the indication of Cu toxicity. Increment of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, and creatinine levels in serum is the indicator of liver and kidney tissue damage and consequently can be used to measure Cu toxicity (El-Masry 2012). High doses of copper sulfate (200 mg/kg) have an adverse effect on their accumulation in large amounts in epididymis, testes, and scrotum of mammals for severe structural alterations and metabolic disruptions which in turn could affect sperm quality that may ultimately lead to infertility (Sakhaee et al. 2012; Tvrda et al. 2015). Reactive oxygen species derived lipid peroxidation is the active members in degradation of polyunsaturated fatty acids present in sperm membrane due to copper toxicity (Abdul-Rasheed, 2010; Tvrda et al. 2011). Antioxidant enzymes, SOD and CAT, are playing a vital role in sperm quality parameters including motility, viability, and sperm concentration (Tvrda et al. 2015). In the testis, heat shock proteins (HSPs) are essential for spermatogenesis and also guard cells from environmental contaminants. HSPs are induced by oxidative stresses and accountable in repair and defensive mechanisms in several tissues. HSP-70 plays a significant role in the testis and functions as a spermatogenic shaperone during germ cell differentiation and as a defensive protein in the process of stress-

induced Leydig cell apoptosis (Rockett et al. 2001; Koeva et al. 2005).

L-carnitine is an important quaternary amine (β-hydroxy-γ-trimethylaminobutyrate) involved in fatty acid metabolism and transports the chains of fatty acids into the mitochondrial matrix. Thus allowing the cells to break down fat and get energy from the stored fat reserves which are also the key fuel source for tissues function indicating its relative significance (Kelly 1998; Pekala et al. 2011). This process is vital in binding/removing abnormal organic acids in several organic acidemias and explains the secondary carnitine deficiency that can result from them (Longo et al. 2006). The major exogenous dietary source of L-carnitine is from meat, fish, poultry, and milk, and also generated endogenously in the liver by methylation of lysine and methionine in the presence of iron, ascorbic acid, niacin, and vitamin B_6 (Ng et al. 2004). L-Carnitine demonstrates a broad array of biological activities including anti-inflammatory, neuroprotective, cardioprotective, gastroprotective, cytoprotective, and antiapoptotic properties (Tarladacalisir et al. 2009; Sayed-Ahmed 2010). L-carnitine is extremely accrued in the male reproductive system, especially in the epididymis. It is involved in male gamete maturation (Ng et al. 2004), production of energy for sperm motility, DNA repair (Garcia et al. 2005), germ cell recovery (Tarladacalisir et al. 2009), and Sertoli cell metabolism (Palmero et al. 2000).

Attempt has been made to prevent the occurrence of the copper poisoning by dietary supplementation with molybdenum and sulfate but these supplements induced a copper deficiency state (Sakhaee et al. 2012). The utilization of L-carnitine and its derivatives in therapy has been recommended for treatment of male infertility, and numerous studies have been published in human (Lenzi et al. 2003, 2004; Seo et al. 2010; Pekala et al. 2011) and animal (Ramadan et al. 2002; Stradaioli et al. 2004; Tarladacalisir et al. 2009; Alshabanah et al. 2010; Cabral et al. 2014) that signify promising L-carnitine application. However, no reports so far on the ameliorative role of L-carnitine against copper-induced testis seminiferous tubules damage in rats and further the mechanisms by which L-carnitine recuperates male fertility are yet to comprehend. In the present study, we hypothesized that L-carnitine therapy (50 and 100 mg/kg) recovers adverse effects of chronic administration of copper (200 mg/kg oral treatment for 30 days) on body and organs weight, food and water consumption, hepatic and renal functions (serum ALT, AST, ALP, urea, and creatinine levels), spermatogenesis, testis histomorphology, histomorphometrics, sperm parameters (sperm concentration, viability, motility, daily sperm production, sperm transit time, and sperm head and tail abnormalities), sperm DNA fragmentation, testis lipid peroxidation, serum testosterone and antioxidant enzymes (superoxide dismutase and glutathione-S-transferase), and immunohistochemical expression of heat shock protein, HSP 70.

Materials and methods

Laboratory animals and ethical permit

Sixty adult male Wistar albino rats (3 months old) weighing 170–200 g were acquired from the Animal Care Facility at the Department of Zoology, Mizoram University, Aizawl, Mizoram, India and were maintained on rat standard pellet diet (Pranav Agro Industries, Maharashtra, India). Food and sterile filtered water were available ad libitum throughout the experiment. The rats were housed in clean filter-top polypropylene plastic cages (40 × 30 × 15 cm) under a well regulated 12-h light, 12-h dark cycle at a controlled temperature of 25 ± 1 °C and relative humidity between 45 and 55% air conditioned room. All animal experiments were carried out in compliance with the Guide of the Institute of Laboratory Animal Resources, National Research Council, Washington (Clark et al. 1996) and the institutional guidelines for the care and use of experimental animals approved by the Committee on the Ethics of Animal Experiments of the Mizoram University Animal Ethical Committee (MZUAEC), Mizoram University, Aizawl, Mizoram, India (University of Mizoram, Ethics permit No. MZUIAEC/29/03/2016-17/13/ZOO/SLS).

Study design and sample collection

A total of 30 mature (3 months old, 170–200 g) male Wistar albino rats were scrutinized rigorously and weighed before the beginning of experimentation. They were arbitrarily divided into six groups (G1–G6) and each group contained five rats. Each of the animals in the group was assigned a number by a concentrated solution of eosin stain on their dorsal body (Korkmaz et al. 2010). A single dose of copper sulfate (200 mg/kg, $CuSO_4{\cdot}5H_2O$, Sigma-Aldrich, St. Louis, MO) was used for inducing copper toxicity in rats due to its deleterious effects on the morphology of testes which appeared within 2 weeks as reported by Babaei and Abshenas (2013) and Kumar et al. (2015).

Group 1 (G1, control group, *n* = 5) Each rat of this group neither received $CuSO_4$ nor L-carnitine but received 0.9% saline orally as placebo along with regular diet and sterile filtered water for 30 days to obtain their morphological, biochemical, and histopathological parameters as the reference comparable values.

Group 2 (G2, $CuSO_4$ group, 200 mg/kg, *n* = 5) Each rat was gavaged orally with $CuSO_4$ at a daily dose of

200 mg/kg b.w./day dissolved in 0.9% saline water for 30 days, respectively.

Groups 3 and 4 (G3 and G4, L-carnitine alone group, 50 and 100 mg/kg) (5 rats/group) These two group rats were gavaged orally with L-carnitine at a daily dose of 50 and 100 mg/kg b.w./day dissolved in 0.9% saline water for 30 days, respectively.

Groups 5 and 6 (G5 and G6, $CuSO_4$ 200 mg/kg plus L-carnitine 50 and 100 mg/kg groups, respectively) (5 rats/group) These two group rats were gavaged orally with $CuSO_4$ 200 mg/kg b.w. plus L-carnitine at a daily dose of 50 and 100 mg/kg b.w./day dissolved in 0.9% saline water for 30 days, respectively.

After exposure to individual and combined $CuSO_4$ and L-carnitine for 30 days, the rats of all groups were anesthetized with light ether anesthesia and venous blood samples were obtained from them by means of capillary glass tubes from the retroorbital venous plexus as described by Nemzek et al. (2001) for the separation of serum samples. Serum samples were stored at − 20 °C until further use for biochemical analysis. All rats were sacrificed by decapitation, the reproductive organs were removed by laparotomy, and testes were decapsulated rapidly and stored in − 80 °C until used for all assays, except for the histology and immunohistochemistry for which the testes were fixed in the Bouin's fixative for 24 h.

Body and organs weights, food and water consumption, liver and kidney tissue damage assays

The body weight (G1–G6 groups, $n = 5$/group) of each rat was measured daily for 30 days. Food and water consumption were estimated by weighing the amount of food and water provided daily to each cage of rats. Data on body weight and food and water consumption were pooled and represented as mean ± standard error. After 30 days treatment, all rats were anesthetized (90 mg/kg ketamine) and were sacrificed by cervical dislocation. The liver, kidney, and reproductive organs (testis, epididymis, seminal vesicle, and vas deferens) were collected and weighed. The serum concentration of AST, ALT, total protein (TP), ALP, urea, and creatinine (CR) were determined by following standard methods (Cheesbrough 2009).

Testis and epididymal sperm assessment

The reproductive organs (testis parenchyma, epididymides, and caudae and caput epididymis) of each rat were separated immediately after euthanasia, cleaned and minced in ice-cold phosphate-buffered saline (PBS, 1:2 *w*/*v*, pH 7.4; 37 °C), and squeezing it gently to obtain the fresh undiluted semen in a clean Petri dish and incubated at 37 °C for half an hour for liquefaction to proceed to the following examinations:

Sperm quality analysis: sperm concentration, motility, morphology, and viability

Sperm samples were acquired from each group at the end of 30 days. The testes and epididymis were gently excised and weighed and the cauda and the caput part of epididymis were isolated and placed in an Eppendorf tube containing 500 μL of PBS. The tissue of cauda and caput epididymis portions was minced by using sharp scissors to release sperms. Sperm quality was determined by three parameters: sperm concentration, motility, morphology, and viability (World Health Organization 1999).

Sperm concentration

Sperm concentration was analyzed using the hemocytometer method (World Health Organization 1999). Sperm suspensions from each treatment group of testis parenchyma and caudal and caput epididymis were diluted to 1:20 with spermicidal solution (4 g sodium bicarbonate plus 1 g phenol in 100 mL distilled water) and transferred into microcentrifuge tubes. The dilution was made using a white blood cell pipette. The sperm suspension from each treatment group was drawn to the 0.5 mark halfway up the stem and the spermicidal solution subsequently to the 11 mark at the top of the bubble chamber. The preparation was then thoroughly mixed and one drop of it was added to both sides of the hemocytometer. The sperms were allowed to settle optimally by keeping the hemocytometer in a humid chamber for 30 min. The humid chamber was constructed by placing a wet sponge inside a fairly air tight ice box. The number of sperm was counted in four corner squares of the hemocytometer with improved doubles Neubauer ruling under a light microscope (× 40 magnifications, Binocular, Olympus CX41, Tokyo, Japan). When sperm crossed the lines of the grid, only those at the top and right hand sides of the squares were counted. Sperm on both sides of the hemocytometer were counted and the average number was recorded. The sperm concentration was calculated as average no. of sperm counted (N) × multiplication factor (10,000) × dilution factor (20) and expressed as $\times 10^6$ sperms/mL ($N \times 0.2 \times 10^6$ sperm).

Sperm motility

Sperm motility was analyzed by placing a drop of sperm suspension (10 μL) from epididymis on a clean glass slide and covers with a cover slip and allowed it to stand for about a minute. The slide was examined under light microscope at × 40 magnifications and motility was scored from 50

different fields. Sperm showing any degree of movement are considered motile and scored 200 sperm/group (motile as well as immotile). Sperm motility was calculated as follows: motility (%) = {[No. of motile sperm/Total number of sperm] × 100} and expressed as the percent motility (World Health Organization 1999).

Sperm viability

Epididymal sperm viability (percent of viable sperm) was assessed by the supra-vital staining technique. One drop of well-mixed liquefied sperm suspension was placed on a clean glass slide on which a drop of eosin-nigrosin stain (5% eosin Y plus 10% nigrosin, 1:1) was added and mixed well. A thin smear was made from this mixture by pipetting 10 μL of mixture onto a clean glass slide and allowed to air dry. The prepared slide was covered with cover slip and examined. Pink-stained dead sperms and unstained live sperms were counted (200 sperm/group) under the light microscope at × 40 magnifications. The viability of sperm was expressed as the percent of viable sperm and computed as viability (%) = {[No. of viable unstained sperm/Total No. of unstained live and pinkish stained dead sperm] × 100} (World Health Organization 1999).

Sperm morphology

For evaluation of sperm morphology, the same preparations (testes parenchyma and cauda and caput portions of epididymis) as used for sperm viability test were examined (Filler 1993). Two hundred normal and abnormal sperm were counted from different fields for each group of rat semen smear. The abnormalities were categorized by the percentage of head and tail abnormalities of sperm considering the following types of modifications: (i) the head shape, including lighter or accentuated curvature; (ii) intermediate piece defects resulting in untied (isolated) heads; (iii) small heads; (iv) lack of usual hook; (v) banana, amorphous, or two heads; (vi) head larger/smaller than normal sperm (macro/micro cephalic); (vii) tail defects, including separated flagellum, flagellum torso, flagellum ball and short, folded, spiral twisted, coiled, or broken tails (Zemanova et al. 2007; Slivkova et al. 2009; Roychoudhury et al. 2016). The percent abnormality = {[No. of abnormal sperm/Total no. of sperm] × 100}.

Daily sperm production, sperm production efficiency, and sperm transit time in the epididymis

The left and right testes of rats were excised from the each treatment group and were homogenized in 5 mL of NaCl 0.9% containing Triton X-100 0.5% and sonicated for 30 s. After a 10-fold dilution, one sample was transferred to Neubauer chambers (four fields per animal) and the late spermatids (stage 19) were counted. Homogenization-resistant step 19 spermatids and sperm from both caput/corpus and cauda epididymis were scored as previously described (Robb et al. 1978). The number of homogenization-resistant spermatids obtained was divided by 6.1 to calculate the daily sperm production (DSP), which refers to the number of days that these spermatids are present in the seminiferous epithelium ($\times 10^6$ sperms/testis/day). The daily sperm production relative to testis weight (DSPr) or sperm production efficiency was calculated by dividing DSP with the testis weight and expressed as $\times 10^6$ sperms/testis/day/g. The sperm transit time through the epididymis (cauda and caput) was determined by dividing the number of sperm in each portion by the DSP (Robb et al. 1978).

Sperm chromatin and DNA evaluation

DNA integrity and chromatin condensation assessments were assessed by standard cytochemical technique including Acridine Orange Test. Acridine orange is a metachromatic fluorescence probe for demonstration of degree of sperm nuclear DNA susceptibility to in situ acid-induced denaturation by distinction between normal native double-stranded DNA (green fluorescent) and abnormally denatured single-stranded DNA (orange/red fluorescent) (Talebi et al. 2012). Briefly, the sperm suspensions of epididymis were prepared for each treatment group as described in sperm quality analysis. A drop of sperm suspension was placed on a clean glass slide and accordingly a thin smear was prepared for each treatment group. After drying the sperm smear, it was fixed in Carnoy's solution (methanol and glacial acetic acid, 3:1) for 5 h or overnight. Then, the slides were washed in distilled water and allowed to air dry for 5–10 min before staining. The sperm smear was stained for 5 min in acridine orange freshly prepared solution (10 mL of 1.0% acridine orange in distilled water was added to a mixture of 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M disodium hydrogen phosphate, pH 2.5) kept in the coupling jar. After 5 min staining, the slide was gently rinsed in distilled water and mounted with distilled water. Smears were assessed on the same day to determine the sperm DNA damage by computing 200 sperms under a fluorescence microscope with × 40 magnifications (Leica DM 2500, model-DFC 450C, Leica Microsystems, Wetzlar, Germany) with excitation of 450–490 nm. Sperm with normal DNA fluoresce green and those abnormal DNA fluoresce yellow or red. Scoring of DNA normality was done immediately after staining (Tejeda et al. 1984; Talebi et al. 2011). DNA fragmentation index (DFI), which is the ratio of abnormally denatured single-stranded DNA (orange/red fluorescent, AO^+) over the sum of double-stranded DNA (green fluorescent, AO^-) and single-stranded DNA (orange/red fluorescent, AO^+). The results of the DFI were expressed as percentages

and considered normal when they were below 30% (Sergerie et al. 2005).

Testis histology, damage score, and histomorphometrics

Histopathological observation

The left testis of each treatment group animal were collected, cut across the midline, and placed in Bouin's fixative solution for 24 h. The tissue pieces were then washed with lithium carbonate in 70% ethanol for 6 h. Then, testes were trimmed, dehydrated through ascending grades of ethanol (50, 70, and 90%) and absolute ethanol for about 1 h each for two times followed by xylene treatment, infiltration with molten paraffin wax, embedded in the paraffin blocks, and placed in a refrigerator until sectioning. Thick sections (5 μm) were obtained by using a rotary microtome (Leica, model RM2125 RTS) and were moved to a water bath maintaining a temperature of 45–50 °C. Folds in the acquired sections were eliminated using a camel hairbrush. The sections were transferred to the glass slides coated with albumin. For hematoxylin and eosin staining, the slides were placed in an oven to warm them, and the slides were then hydrated using descending grades of ethanol: 100, 80, 70, and 50%, each for 1 min. Then, the slides were washed with distilled water for 2 min. The slides were then placed in hematoxylin (stains negatively charged nucleic acids (ribosomes and nuclei) a deep purplish-blue color) dye for 8 min, dipped in acidic alcohol, and neutralized in ammoniacal water. Subsequently, the slides were counterstained in eosin (an acidophilic dye that colors the cytoplasmic material orange-pink) for 16 min and then dehydrated using ascending grades of ethanol. After staining, the slides were cleaned one by one in xylene, mounted with DPX, and covered with cover slips that were placed gently to avoid air bubbles (Bancroft and Gamble 2002). The testis tissues were evaluated for histopathological alterations under standard light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) following the protocols of Bancroft and Gamble (2002).

Seminiferous tubule damage scoring and evaluation of spermatogenic recovery

Histopathological alterations in the seminiferous tubules were randomly sampled in 100 tubular sections per treatment group. The level of damage was categorized by a crescent score between 1 and 4 and expressed in percent score as follows: 1 = 0–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100% (Oliva and Miraglia 2009), with some modifications. The specific histopathological alterations scored were as follows: intraepithelial vacuolation, cellular debris, sloughed cells into the tubular lumen, and different levels of germ cell depletion, which were determined based on the number of seminiferous epithelium layers and on the cellular types found in this epithelium (Cabral et al. 2014).

The level of spermatogenesis after $CuSO_4$ and CAR treatment was assessed by determining repopulation index in rats killed at 30 days after treatment. The recovery of spermatogenesis was evaluated based on differentiation of tubules which contained three or more cells that had attained the type B spermatogonia (dense clumps of heterochromatin around the periphery of the nucleus, while type A characterized and differentiated by pale staining nucleus with a fine dusty distribution of heterochromatin) stage or later. The tubule differentiation index (TDI) was computed as the percent of tubules showing differentiation using 100 seminiferous tubules in one section from each rat, thus 600 tubules for each treatment group (Meistrich and van Beek 1993).

Mean seminiferous tubule diameter, Johnsen's testicular biopsy score, and quantification of germ cells and Sertoli cells

Nine slides (3 parts × 3 slides) from upper, mid, and lower parts of right testis were prepared to calculate mean seminiferous tubule diameter (MSTD), Johnsen's testicular biopsy score (JTBS), and number of spermatogenic cells per tubule. All the observations were made at random order in 100 tubules of the right testis tissue sections under blindfold conditions. Only cells with whole nuclei were considered for counting. Same testis tissue sections were used to compute MSTD, JTBSs, and number of germ cells and Sertoli cells per tubule. MSTD was measured on H&E-stained paraffin sections at × 100 magnification under light microscope (Olympus CX41) using a micrometric ocular lens. Testis injury and disorder in spermatogenesis were appraised histopathologically using JTBS criteria (Johnsen 1970). The scoring grades for JTBS were as follows: score 10, complete spermatogenesis with regular tubules; score 9, many sperms, irregular germinal epithelium; score 8, few sperms; score 7, no sperms, many spermatids; score 6, few spermatids; score 5, no sperm or spermatids; score 4, few spermatocytes; score 3, presence of spermatogonia; score 2, presence of Sertoli cells; score 1, no cells. JTBS was computed as sum of all scores in each treatment group/total number of seminiferous tubules. A decrease in the average value of JTBS was assessed as an impairment of spermatogenesis. In addition, average number of germ cells (spermatogonia, spermatocytes, and spermatids) and Sertoli cells/tubule was examined and quantified. Only Sertoli cells exhibiting distinctive morphologic features and clear nucleolus were quantified. Since the number of germ cells in tubular section at stages I–VIII is different from those at stages IX–XIV, 50 tubules at stage I–VIII and 50 tubules at stage IX–XIV per rat were counted (Roy et al. 2017).

Immunohistochemistry

Immunohistochemical staining was performed according to the avidin-biotin-peroxidase complex (ABC) method (Hsu et al. 1981). Embedded testes in paraffin wax were sectioned at 5 μm and de-waxed with xylene followed by rehydrated through a graded series of ethanol to a final wash in water. Tissue sections were treated with 3% H_2O_2 in methanol for 10 min to inhibit endogenous peroxidase activity. The tissue sections were washed in distilled water for 10 min and were incubated with normal goat serum (Lot#D2514; Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500 dilution in PBS) for 1 h to block non-specific binding of antibodies. The testis tissue sections were incubated with heat shock protein antigen (HSP 70) primary antibody (mouse monoclonal HSP70 (3A3) antibody, FL-261, Cat# SC-32239, Lot# H0613, Santa Cruz Biotechnology, St. Louis, MO, USA) diluted 1:200 for 12 h at 4 °C. The sections were then washed and incubated with the biotinylated donkey anti-goat IgG-HRP conjugated secondary antibody (SC-2020, Lot# D2514; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. The sections were incubated again with the chromagen substrate (0.1% 3,3′diaminobenzidine tetra hydrochloride (DAB) in 0.5 M Tris 7.6 and 0.01% H_2O_2) for 10 min and counter-stained with hematoxylin for 30 s as per the manufacturer's instructions. Slides were analyzed under a light microscope (Leica DM2500) with a digital camera (model—DFC 450C) (Leica Microsystems, Wetzlar, Germany) and photographed. To test the specificity of the immunoreactivity in the negative control section, the primary antiserum was replaced with 1% normal rabbit serum.

Serum testosterone assay

The serum testosterone was measured by using human enzyme-linked immunosorbant assay (ELISA) kit (cat# AA E-1300, Labor Diagnostika Nord, GmbH, Am Eichenhain, Nodhorn, Germany) as per the instructions of the manufacturer. In brief, for testosterone assay, standard, control, or samples (50 mL) were added in each well of the ELISA plate. Subsequently, conjugate solution (100 mL) and testosterone antiserum (100 mL) were added to each of these wells. The ELISA plate was then incubated with mild shaking (500–700 rpm) in an incubator shaker (model—SLM-INC-OS-250, Shalimar Specialty Chemical Ltd., Bangalore, Karnataka, India) at room temperature for 1 h. The wells were then aspirated and washed five times with a wash solution. Then tetramethyl benzidine (TMB) substrate (cat# AA E-0055, Labor Diagnostika Nord, GmbH, Am Eichenhain, Nodhorn, Germany) chromogen solution (100 mL) was added to each well and the plate was incubated at room temperature for 30 min. Finally, stop solution (0.2 M sulphuric acid, 100 mL) was added and the absorbance was taken at 450 nm using a microplate reader (SpectraMax M2e, microplate reader, Molecular Devices, Sunnyvale, CA).

Processing of testes tissues, lipid peroxidation, and antioxidant enzyme assays

The reproductive organs (testis, epididymis—caput and cauda portions) were harvested from each treatment group, and homogenized with ice-cold Tris-EDTA suspension buffer in a glass homogenizer to produce 10% (*w*/*v*) homogenate. The homogenates were filtered and centrifuged for 30 min at 10,000 × *g* at 4 °C and the supernatants were frozen at − 80 °C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the Lowry method (Lowry et al. 1951).

The lipid peroxidation product (LPP) formation in the testes tissues was estimated by measuring the thiobarbituric acid reactive substances and quantified as malondialdehyde (MDA) formed (nmol/mg protein) by using 1,1,3,3-tetramethoxypropane as the standard following the spectrophotometric method of Ohkawa et al. (1979). Testes tissue homogenates (10%) were prepared in potassium chloride (1.5%). The testes tissue protein contents were precipitated by adding 2.5 mL of trichloroacetic acid (TCA) to 1 mL of the homogenate. The precipitated proteins were centrifuged at 2500 × *g* for 10 min at 4 °C. The resultant pellet was suspended in 2.5 mL of 0.05 M sulfuric acid and to this, 3 mL of 2 M thiobarbituric acid (TBA) was also added. The whole suspension was incubated with TBA for 30 min in boiling water bath (100 °C). The contents were cooled to room temperature, and pink color was extracted into 4 mL of *n*-butanol. The color obtained was read at 530 nm using a spectrophotometer (Eppendorf UV-9200) against the blank.

Testicular SOD was assayed by the method of Asada et al. (1974) which involves the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at pH 8.0. SOD activity was defined as the quantity of superoxide dismutase required to produce 50% inhibition of photochemical reduction of NBT. Briefly, the reaction mixture containing 100 μL of phenazene methosulfate (PMS, 0.06 mg/mL), 300 μL of NBT (2.5 mg/mL), and 200 μL of NADH (0.6 mg/mL of *n*-butanol) was incubated at 30 °C for 90 s. Reaction was stopped by adding 1 mL of acetic acid and after that 4 mL of *n*-butanol was added. The absorbance was read at 560 nm against a blank using UV–Vis spectrophotometer. The activity was expressed as micromole/milligram protein.

Glutathione-S-transferase (GST) activity was quantified by checking formation of a thioether bond between GSH and 1-chloro-2,4-dinitrobenzene (CDNB as a substrate, Sigma-Aldrich, St. Louis, MO, USA) using a spectrophotometer (Warholm et al. 1985). The formation of the product of 1-chloro-2,4-dinitrobenzene, S-2,4-dinitro-phenyl glutathione, was monitored by measuring the net increase in absorbance

at 340 nm against the blank. The enzyme activity was calculated using the molar extinction coefficient of = 9.6 M/cm and expressed as nanomole/milligram of protein.

Univariate and multivariate statistical analyses

All data were expressed as mean ± SEM. Normality distribution of the variables was tested using one sample Kolmogorov-Smirnov test. Differences in measured parameters among the groups were analyzed by one-way analysis of variance (ANOVA) test due to normal distribution. The results were analyzed by one-way ANOVA followed by Tukey's test for post hoc comparisons using SPSS for Windows (SPSS, Inc. Chicago, IL, USA, ver. 20.0). Correlation and regression analysis were made to find the relationship (i) between serum testosterone levels and JTBS, MSTD, sperm number in cauda, and DSP, respectively, and (ii) between oxidative stress (lipid peroxidation) and serum testosterone, sperm motility, DSP, sperm number in cauda, and sperm head and tail abnormalities, respectively.

Multivariate analysis of variance (MANOVA) including principal component analysis (PCA), hierarchical cluster analysis (Euclidean distance measure), and detrended correspondence analysis (DCA) was performed using PAST (version 1.86b) software (Hammer et al. 2001). PCA was performed to investigate and ordinate the relationship between supplementation of L-carnitine dose and their remedial measures of copper-induced clinical complications in the rat testis. Thus, the axes derived correspond to gradients of modulatory action of L-carnitine and copper toxicity impairment in testes and dysfunction of spermatogenesis in relation to morphological, histopathological, histomorphometric, hormonal, biochemical, immunohistochemical, and sperm quality assessments. Hierarchical cluster analysis was carried to classify the uniqueness and differences of the L-carnitine treatment groups and their impact on restoration of copper-induced testicular toxicity using Bray-Curtis distance measure. In addition, L-carnitine treatment groups were classified according to dose and their modulation effects on revival of copper-induced testis toxicity by DCA (Hammer et al. 2001).

Results

Clinical assessments, body and organs weight, food and water consumption, and hepatic and renal functions

Clinical manifestations of copper toxicity

The $CuSO_4$ (200 mg/kg) administered rats (G2 group) showed the following copper toxicity signs and symptoms as follows: (1) slow activity; (2) paleness of mucous membranes of eyes and pads extremities; (3) rough dried skin with alopecia especially in the abdominal region; (4) enlargement of liver with dark spots and its borders were swollen, friable, and yellow in color; (5) bilateral enlargement of kidney with a dark brown in color; (6) congested spleen and enlarged in size; (7) swollen, congested, and edematous brain; (8) flabby, enlarged, and congested heart; (9) thickened stomach wall with corrugated mucosa; and (9) rough and dry skin coat. No signs of morbidity or mortality or adverse effects were noticed either in the L-carnitine alone treated rats (G3 and G4 groups, 50 and 100 mg/kg) or L-carnitine + $CuSO_4$ treated rats (G5 and G6 groups, 50 and 100 mg/kg + 200 mg/kg) evidencing the potential benefits of L-carnitine supplements in alleviation of clinical manifestations of copper toxicity.

Body weight, food and water consumption, and serum biochemical parameters

A significant decrease ($p < 0.05$) in body weight (99.64 vs 106.09 g), food consumption (18.62 vs 26.41 g), and water intake (30.28 vs 51.45 mL) were observed in $CuSO_4$ treated rats (G2 group, 200 mg/kg) compared to controls (G1 group) (Table 1). No significant differences were observed between L-carnitine supplemented treatment groups (G3–G6 groups) and control groups, respectively in terms of body weight, food and water consumption (Table 1).

A significant increase in serum ALT (81.65 vs 48.08 IU/L), AST (156.82 vs 88.25 IU/L), ALP (230.54 vs 148.16 IU/L), urea (12.65 vs 7.45 mmol/L), and creatinine (80.61 vs 48.25 μmol/L) levels were noticed in G2 group ($CuSO_4$, 200 mg/kg) while these parameters were found to be within the normal reference ranges in the L-carnitine alone (G3 and G4 groups) and L-carnitine + $CuSO_4$ (G5 and G6 groups) treatment. This observation confirmed the good health and normal functioning of the liver and kidney of the L-carnitine supplemented treatment groups (Table 1).

Liver, kidney, and reproductive organs weights

The absolute and relative weights of the liver and kidney were significantly decreased ($p < 0.05$) in $CuSO_4$ treated G2 group rats as compared with the G1 control group. While, L-carnitine alone treated rats (groups G3 and G4) as well as L-carnitine + $CuSO_4$ administered rats (groups G5 and G6) showed no statistically ($p > 0.05$) significant alterations in the organs weights in comparison with the controls indicating the rejuvenation and restoration ability of L-carnitine supplement (Table 2).

A significant reduction in absolute and relative reproductive organs weight (testes, epididymis, seminal vesicle, and vas deferens) was noticed in $CuSO_4$ treated rats (G2 group) in comparison with the G1 control group indicating testicular atrophy and damage. However, L-carnitine alone (G3 and G4 groups) and in combination with $CuSO_4$ (G5 and G6 groups)

Table 1 Effect of L-carnitine on body weight, food consumption, water intake, liver and kidney function tests following 30 days chronic administration of copper sulfate to Wistar albino rats

Parameters	Treatment group						$F_{5,29}$ value
	Control (group 1—G1)	$CuSO_4$ (200 mg/kg) (group 2—G2)	L-carnitine (50 mg/kg) (group 3—G3)	L-carnitine (100 mg/kg) (group 4—G4)	$CuSO_4$ + L-carnitine (50 mg/kg) (group 5—G5)	$CuSO_4$ + L-carnitine (100 mg/kg) (group 6—G6)	
Initial body weight (g)	100.77 ± 3.07	104.68 ± 5.41	101.51 ± 2.15	102.71 ± 2.94	108.96 ± 4.16	105.38 ± 2.99	
Final body weight (g)	106.09 ± 1.55a	99.64 ± 8.16b	106.63 ± 2.31a	107.35 ± 3.30a	114.29 ± 3.77a	110.37 ± 0.78a	12.127
Increase/decrease in body weight (%)	5.22 ± 1.26a	− 4.95 ± 0.95b	5.11 ± 1.60a	4.94 ± 1.91c	4.96 ± 1.16a	4.92 ± 1.78a	47.013
Food consumption (g)	26.41 ± 1.75a	18.62 ± 1.38b	27.18 ± 1.38a	29.91 ± 1.86a	27.85 ± 1.25a	28.60 ± 1.10a	7.362
Water intake (mL)	51.45 ± 1.66a	30.28 ± 1.08b	54.65 ± 1.20a	55.15 ± 1.75a	50.75 ± 1.35a	52.11 ± 1.81a	38.946
Aspartate aminotransferase (IU/L)	88.25 ± 9.45a	156.82 ± 7.25b	89.36 ± 7.21a	87.44 ± 7.38a	92.26 ± 6.75a	85.10 ± 8.10a	11.423
Alanine aminotransferase (IU/L)	48.08 ± 3.90a	81.65 ± 6.08b	47.00 ± 4.85a	49.35 ± 8.91a	52.01 ± 7.16a	50.62 ± 5.75a	4.420
Alkaline phosphatase (IU/L)	148.16 ± 9.45a	230.54 ± 9.15b	150.25 ± 7.18a	155.05 ± 6.35a	156.28 ± 5.24a	153.16 ± 4.38a	19.682
Urea (mmol/L)	7.45 ± 1.66a	12.65 ± 1.95b	6.95 ± 1.55a	8.05 ± 1.90a	8.45 ± 1.88a	6.16 ± 1.05a	13.722
Creatinine (μmol/L)	48.25 ± 1.25a	80.61 ± 1.05b	49.55 ± 1.15a	50.71 ± 1.62a	52.65 ± 1.58a	50.36 ± 1.92a	72.765

Data are expressed as mean ± standard error mean ($n = 5$). The relative organ weight was calculated by use of the formula: [(organ weight/bodyweight) × 100]. g, gram; mg, milligram; kg, kilogram. Data marked with different letters (a, b) are statistically significant ($p < 0.0001$) if they do not share the same letters

significantly improved the absolute and relative reproductive organs weight when compared with the rats treated only with $CuSO_4$ (G2 group) (Table 2).

Sperm quality analysis

Epididymal sperm motility

Motile sperms of the epididymal contents were observed to be high in the control G1 group rats (64.36%) while a significant reduction in the number of motile sperms was noticed ($p < 0.05$) with $CuSO_4$ treated G2 group rats (38.48%). Most of the sperm were observed to be dead in $CuSO_4$ treated G2 group. But, significantly high number of motile sperms was seen in L-carnitine alone (G3 group—64.6% and G4 group—65.93%) and L-carnitine + $CuSO_4$ (G5 group—62.59% and G6 group—63.4%) treated groups in comparison with $CuSO_4$-administered G2 group rats indicating the protective and restoring role of L-carnitine supplement on sperm quality. The frequency of number of motile sperms in L-carnitine supplemented groups (G3–G6) was at par with the control G1 group (Fig. 1a).

Epididymal sperm viability

Oral administration of $CuSO_4$ (G2 group, 200 mg/kg) for 30 days significantly decreased ($p < 0.05$) the percent live viable sperms (26.88%) and proportionately increased the dead sperms (73.12%) in $CuSO_4$ treated G2 group compared to the G1 control group (live sperms, 91.65% and dead sperms, 8.35%). A significant increase ($p < 0.05$) was observed in sperm viability in rats exposed to L-carnitine alone (live sperms: G3 group—90.48%, G4 group—92.77% and dead sperms: G3 group—7.23, G4 group—9.52%) and L-carnitine + $CuSO_4$ groups (live sperms: G5 group—88.62%, G6 group—93.66% and dead sperms: G5 group—6.34%, G6 group—11.38%) compared to $CuSO_4$ treated G2 group (Fig. 1b).

Sperm number in testis, cauda, and caput

Sperm number in the testis (Fig. 1c, d), cauda (Fig. 1e, f), and caput (Fig. 1g, h) was observed in terms of per organ ($\times 10^6$ sperms/mL) and per gram of organ ($\times 10^6$/g) and found a significant decrease ($p < 0.05$) in the $CuSO_4$ treated rats (G2 group—testis: 96.12×10^6 and 98.64×10^6/g; caput: 84.38×10^6 and 544.36×10^6/g; cauda: 84.63×10^6 and 564.17×10^6/g) in comparison with the untreated control rats (G1 group—testis: 154.25×10^6 and 164.09×10^6/g; caput: 221.63×10^6 and 2318.25×10^6/g; cauda: 224.25×10^6 and 2038.64×10^6/g) supported the detrimental effects of copper on the sperm number (Fig. 1c–h). However, L-carnitine coadministration with $CuSO_4$ (G5 and G6 groups) significantly

Table 2 Effect of L-carnitine on absolute and relative weights of liver, kidney, and reproductive organs of male rats exposed to copper sulfate for 30 days

Body weight and organs absolute and relative weights	Treatment group						$F_{5,29}$ value
	Control (group 1—G1)	$CuSO_4$ (200 mg/kg) (group 2—G2)	L-carnitine (50 mg/kg) (group 3—G3)	L-carnitine (100 mg/kg) (group 4—G4)	$CuSO_4$ + L-carnitine (50 mg/kg) (group 5—G5)	$CuSO_4$ + L-carnitine (100 mg/kg) (group 6—G6)	
Liver (g)	4.99 ± 0.12a	3.48 ± 0.10b	4.81 ± 0.09a	4.85 ± 0.12a	4.85 ± 0.16a	4.91 ± 0.06a	27.08
Liver (g/100 g)	3.37 ± 0.15a	2.36 ± 0.08b	3.30 ± 0.09a	3.23 ± 0.11a	3.41 ± 0.05a	3.50 ± 0.10a	4.533
Kidney (mg)	474.78 ± 2.16a	429.29 ± 0.88b	464.55 ± 1.20a	473.20 ± 1.23a	469.74 ± 1.58a	469.56 ± 1.42a	1337.9
Kidney (mg/100 g)	411.60 ± 1.54a	398.41 ± 0.84b	410.91 ± 1.09a	410.22 ± 1.07a	415.20 ± 1.34a	417.37 ± 1.43a	370.03
Testis (g)	0.96 ± 0.01a	0.58 ± 0.00b	1.00 ± 0.05a	1.02 ± 0.07a	0.93 ± 0.01a	0.95 ± 0.01a	31.636
Testis (g/100 g)	0.92 ± 0.01a	0.58 ± 0.00b	0.94 ± 0.04a	0.96 ± 0.06a	0.90 ± 0.01a	0.97 ± 0.01a	25.502
Epididymis (mg)	307.68 ± 1.58a	175.95 ± 1.31b	306.33 ± 0.83a	308.99 ± 1.00a	306.25 ± 0.83a	310.05 ± 0.67a	4455.5
Epididymis (mg/100 g)	295.59 ± 1.52a	126.00 ± 0.94b	293.50 ± 0.76a	296.42 ± 0.90a	291.79 ± 0.70a	303.27 ± 0.67a	7207.0
Seminal vesicle (mg)	615.67 ± 1.38a	467.76 ± 1.47b	615.22 ± 1.32a	616.81 ± 1.20a	612.93 ± 0.94a	618.28 ± 1.37a	4119.4
Seminal vesicle (mg/100 g)	591.47 ± 1.32a	334.97 ± 1.05b	589.34 ± 1.14a	590.84 ± 1.10a	595.58 ± 0.80a	594.99 ± 1.38a	9877.5
Vas deferens (mg)	85.40 ± 1.29a	66.02 ± 1.14b	84.86 ± 0.92a	86.28 ± 1.09a	82.31 ± 0.83a	84.85 ± 0.88a	36.717
Vas deferens (mg/100 g)	82.04 ± 1.24a	47.27 ± 0.82b	82.89 ± 0.79a	82.58 ± 1.00a	81.64 ± 0.70a	83.33 ± 0.89a	169.92

Data are expressed as mean ± standard error mean ($n = 5$). The relative organ weight was calculated by use of the formula: [(organ weight/bodyweight) × 100]. g, gram; mg, milligram; kg, kilogram. Data marked with different letters (a, b) are statistically significant ($p < 0.0001$) if they do not share the same letters

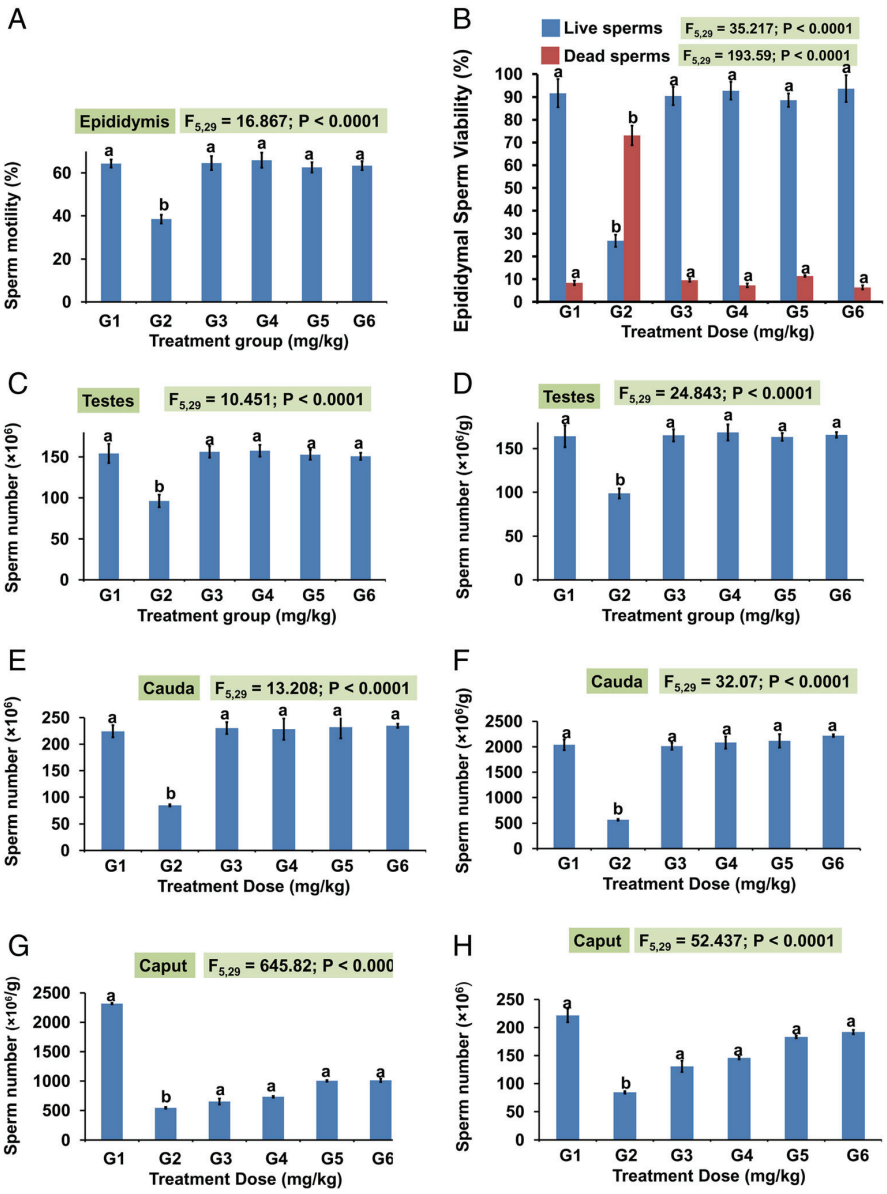

Fig. 1 Effect of L-carnitine on sperm motility, sperm viability and sperm count of adult albino rats exposed to copper sulfate (200 mg/kg) and/or L-carnitine (50 and 100 mg/kg) for 30 days. **a** Epididymal sperm motility (%). **b** Epididymal sperm viability (%). **c** Sperm number in testis ($\times 10^6$ sperms/mL). **d** Sperm number relative to testis weight ($\times 10^6$ sperms/g). **e** Sperm number in cauda ($\times 10^6$ sperms/mL). **f** Sperm number relative to caudal weight ($\times 10^6$ sperms/g). **h** Sperm number in caput ($\times 10^6$ sperms/mL). **i** Sperm number relative to caput weight ($\times 10^6$ sperms/g). Values are mean of five observations ($n = 5$). All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison test. Bar with different letter means statistical significant difference at $p < 0.05$ and similar letters are not significant. G1, control group (0.9% saline); G2, $CuSO_4$ group (200 mg/kg); G3, L-carnitine group (50 mg/kg); G4, L-carnitine group (100 mg/kg); G5, $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg); G6, $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg)

reinstates sperm counts toward the normal untreated control group rats. Moreover, the coadministration of L-carnitine with $CuSO_4$ demonstrated a significant higher count of epididymal (cauda and caput) sperms when compared with the $CuSO_4$ treated rats (G2 group), which confirmed the additional protective role of L-carnitine on copper-induced testicular sperm degeneration (Fig. 1c–h).

DSP and DSPr in testis and sperm transit time in epididymis

DSP per testis (the number of homogenization-resistant step 19 spermatids) and sperm production efficiency (DSPr) were significantly lower (DSP, 13.21 × 10^6 sperms/mL; DSPr, 13.76 × 10^6/g) in $CuSO_4$ treated G2 group rats (Fig. 2a, b) in comparison with those from the G1 control group rats (DSP, 26.90 × 10^6 sperms/mL; DSPr, 21.29 × 10^6/g). On the other hand, L-carnitine coadministration with $CuSO_4$ (G5 and G6 groups) demonstrated a significant increase in DSP (25.14–29.08 × 10^6 sperms/mL) as well as DSPr (21.83–22.39 × 10^6/g) toward the control G1 group level which confirmed its effectiveness in restoring the sperm quality (Fig. 2a, b). In addition, the sperm transit time in the epididymal cauda and caput/corpus regions was significantly lower in the $CuSO_4$ treated G2 group rats (cauda, 6.41 days; caput, 6.39 days) than

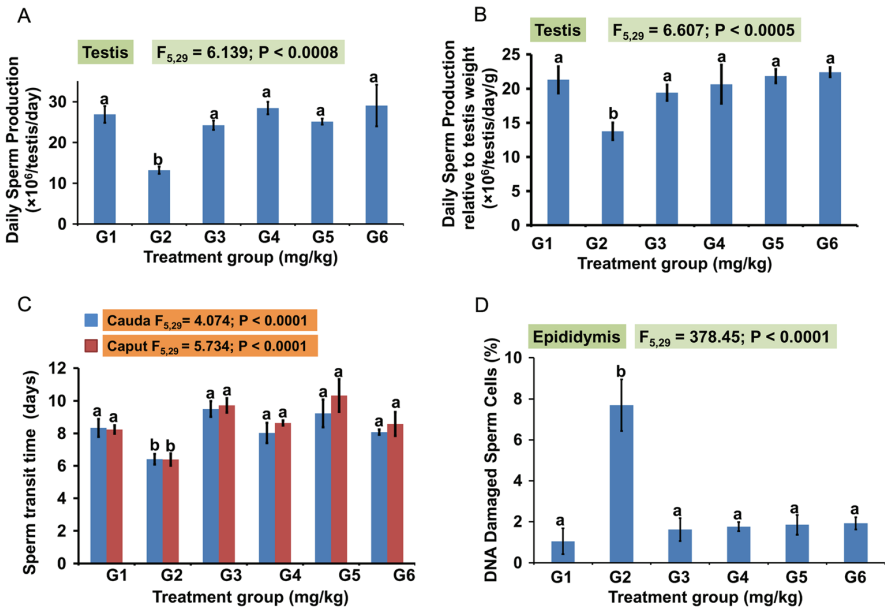

Fig. 2 Modulatory action of L-carnitine on daily sperm production, efficiency of sperm production, sperm transit time, and sperm DNA and chromatin integrity of adult albino rats administered with copper sulfate (200 mg/kg) and/or L-carnitine (50 and 100 mg/kg) for 30 days. **a** Daily sperm production in testis ($\times 10^6$ sperms/testis/day). **b** Efficiency of sperm production in testis ($\times 10^6$ sperms/testis/day/g). **c** Sperm transit time in caput and cauda ($\times 10^6$ sperms/mL). **d** Sperm DNA and chromatin integrity assessment using acridine orange staining (% DNA damaged sperm cells). Values are mean of five observations (n = 5). All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison test. Bar with different letter means statistical significant difference at $p < 0.05$ and similar letters are not significant. G1, control group (0.9% saline); G2, $CuSO_4$ group (200 mg/kg); G3, L-carnitine group (50 mg/kg); G4, L-carnitine group (100 mg/kg); G5, $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg); G6, $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg)

in the control G1 group (cauda, 8.34 days; caput, 8.24 days) while no significant alterations were found in the L-carnitine alone (G3 and G4) and L-carnitine + $CuSO_4$ (G5 and G6) supplement groups rats (cauda—G3 and G4 group: 8.02–9.50 days; G5 and G6 group: 8.07–9.23 days; and caput—G3 and G4 group: 8.64–9.72 days; G5 and G6 group: 8.58–10.32 days) (Fig. 2c).

Sperm head and tail abnormalities

The sperm head and tail abnormalities were monitored to ensure the detrimental effect of copper on sperm. The sperm head and tail abnormalities were increased significantly after copper treatment (G2 group) when compared with the G1 controls (Table 3). The normal sperm phenotypes were significantly decreased to 54.70% in $CuSO_4$ treated G2 group and most of the sperms were observed to be dead (Fig. A.1A). L-carnitine supplemented groups [L-carnitine (G3 and G4) and L-carnitine + $CuSO_4$ (G5 and G6)] significantly restored (97.73–98.37%) this parameter toward the G1 control level (98.20%). The common abnormality encountered during the morphological examination of the sperms was banana heads (Fig. A.1B), two heads (Fig. A.1E, H), small/big head (Fig. A.1G, I), untied head (Fig. A.1B), amorphous head (Fig. A.1D), lack of usual hook (Fig. A.1G), separated flagellum (Fig. A.1I), coiled tails (Fig. A.1C), broken tails (Fig. A.1E), and spiral twisted tails (Fig. A.1D). Within head abnormalities observed in $CuSO_4$ exposure (G2 group), banana head were the predominant form of sperms (17.01%, Fig. A.1B, E, H) followed by head without hook (5.79%, Fig. A.1D, G, H), detached head (5.65%, Fig. A.1B, I), and amorphous head (3.41%, Fig. A.1D, G). Regarding tail abnormalities in $CuSO_4$ treated G2 group, coiled tail forms were observed mostly (10.57%, Fig. A.1C, F) followed by broken tail sperms (3.76%, Fig. A.1E, F, I). All together $CuSO_4$ exposure significantly damaged the sperm morphology and in turn noticed more number of morphologically (G2 group: 32.82% total head abnormalities and 14.85% total tail abnormalities) abnormal sperm (Table 3). L-carnitine + $CuSO_4$ supplementation (G5 and G6 groups) significantly protected the sperm completely from the detrimental effects of $CuSO_4$ as a lower percentage of sperm head and tail abnormalities were evident in both the groups of animals treated with L-carnitine along with $CuSO_4$ (Table 3).

Sperm chromatin and DNA evaluation

In acridine orange staining, the chromatin quality of sperm was assessed according to metachromatic fluorescence

Table 3 Gonadoprotective action of L-carnitine on head and tail sperm abnormalities and DNA fragmentation index in male rats exposed to copper sulfate for 30 days

Sperm morphology	Experimental groups						
	Control (group 1—G1)	$CuSO_4$ (200 mg/kg) (group 2—G2)	L-carnitine (50 mg/kg) (group 3—G3)	L-carnitine (100 mg/kg) (group 4—G4)	$CuSO_4$ + L-carnitine (50 mg/kg) (group 5—G5)	$CuSO_4$ + L-carnitine (100 mg/kg) (group 6—G6)	$F_{5,29}$ value
Assessment of sperm head and tail abnormalities							
Normal phenotypes (%)	98.20 ± 3.49a	54.70 ± 4.05b	98.37 ± 3.28a	98.07 ± 3.12a	97.84 ± 2.16c	97.73 ± 3.14a	280.74
Head abnormalities (%)							
Amorphous head	0.26 ± 0.05a	3.41 ± 0.64b	0.20 ± 0.03a	0.24 ± 0.06a	0.70 ± 0.63a	0.24 ± 0.52a	47.536
Head without hook	0.46 ± 0.06a	5.79 ± 0.65b	0.50 ± 0.12a	0.51 ± 0.07a	0.51 ± 0.85a	0.44 ± 0.54a	45.411
Detached head	0.61 ± 0.09a	5.65 ± 0.59b	0.64 ± 0.06a	0.66 ± 0.06a	0.76 ± 0.64a	0.68 ± 0.74a	152.63
Banana head	0.65 ± 0.06a	17.01 ± 0.82b	0.68 ± 0.29a	0.66 ± 0.06a	0.69 ± 0.71a	0.72 ± 0.61a	151.79
Total of head abnormalities	2.01 ± 0.43a	32.82 ± 0.67b	2.64 ± 0.60a	2.58 ± 0.45a	2.99 ± 0.70a	2.75 ± 0.63a	749.23
Tail abnormalities (%)							
Coiled tail	0.10 ± 0.00a	10.57 ± 0.53b	0.14 ± 0.03a	0.13 ± 0.05a	0.28 ± 0.33a	0.19 ± 0.44a	189.86
Broken tail	0.13 ± 0.00a	3.76 ± 0.45b	0.12 ± 0.00a	0.11 ± 0.05a	0.19 ± 0.04a	0.17 ± 0.58a	28.749
Total of tail abnormalities	0.14 ± 0.00a	14.85 ± 0.63b	0.18 ± 0.02a	0.28 ± 0.15a	0.56 ± 0.55a	0.41 ± 0.57a	157.80
DNA integrity and sperm chromatin condensation assessments—acridine orange staining							
DNA fragmentation index (DFI %)	11.11 ± 0.62a	88.37 ± 5.86b	17.31 ± 1.54a	17.76 ± 2.36a	17.02 ± 1.18a	18.81 ± 1.22a	114.69

Data are expressed as mean ± standard error mean ($n = 5$). Data marked with different letters (a, b) are statistically significant ($p < 0.05$) if they do not share the same letters

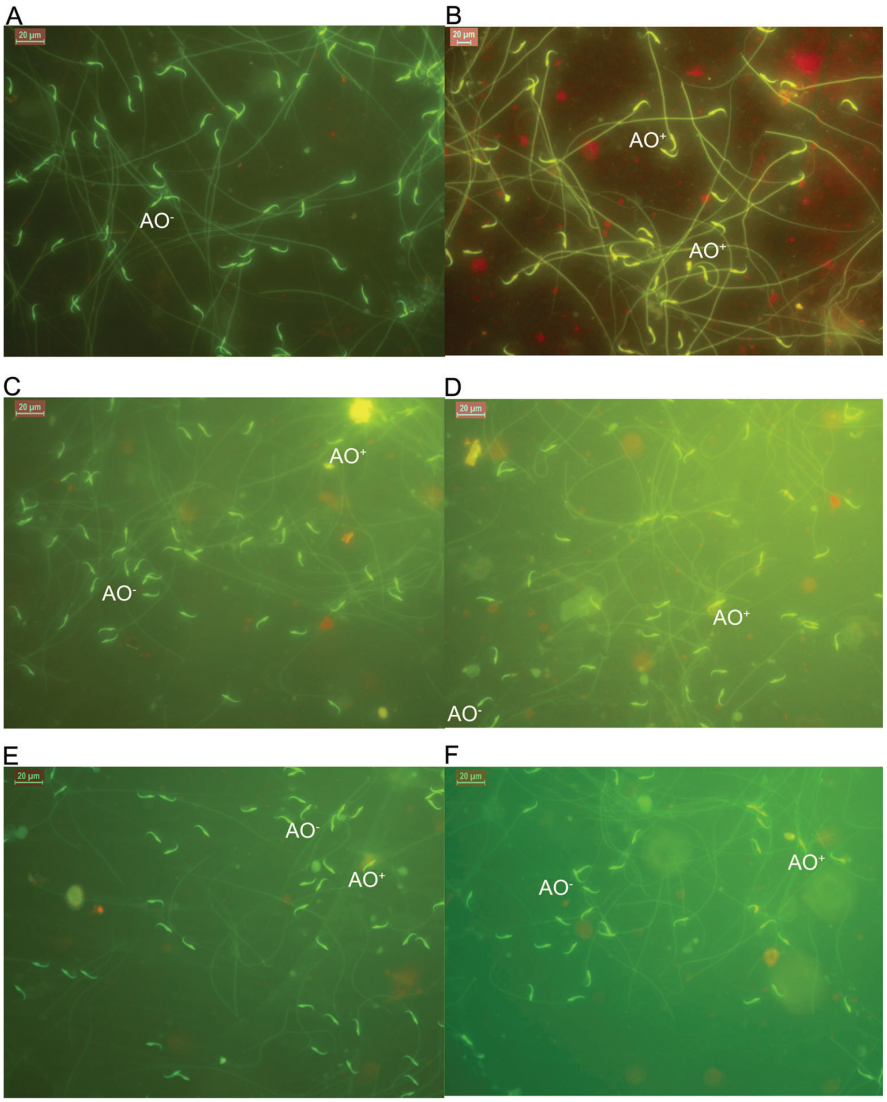

Fig. 3 DNA integrity and chromatin condensation assessments by acridine orange staining for demonstration of degree of sperm nuclear DNA susceptibility to in situ acid-induced denaturation by distinction between native double-stranded DNA (green fluorescent, ***AO***$^{-}$) and denatured single-stranded DNA (yellow/red fluorescent, AO^{+}). **a** Control group (0.9% saline). **b** $CuSO_4$ group (200 mg/kg). **c** L-Carnitine group (50 mg/kg). **d** L-Carnitine group (100 mg/kg). **e** $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg). **f** $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg). Acridine orange staining, ×40 eyepiece magnification, *scale bar* = 20 μm. Frequency of green fluorescent sperms was more in treatment groups **a** and **c**–**f** while group **b** showing more sperms with yellow or red fluorescent, respectively

staining of sperm heads and demonstrated the degree of sperm nuclear DNA susceptibility to in situ acid-induced denaturation by distinction between normal native double-stranded DNA (green fluorescent) and abnormally denatured single-stranded DNA (orange/red fluorescent) (Fig. 3). In acridine orange staining, the presence of normal native double-stranded DNA (green fluorescent, AO^{-}) was observed to be high in the treatment groups of untreated G1 control (Fig. 3a), L-carnitine alone (G3 and G4 groups, Fig. 3c, d), and L-carnitine + $CuSO_4$ (G5 and G6 groups, Fig. 3e, f) while reverse trend was noticed in $CuSO_4$ treated rats (G2 group, Fig. 3b), i.e., detection of high number of abnormally denatured single-stranded DNA (orange/red fluorescent, AO^{+}). $CuSO_4$ treatment significantly increased the sperm DNA damage (denatured single-stranded DNA, orange/red fluorescent) to the tune of 7.69% while the percent sperm DNA damage was significantly decreased to 1.62–1.92% in L-carnitine alone (G3 and G4 groups) and L-carnitine + $CuSO_4$ (G5 and G6 groups) treated rat groups, respectively (Fig. 2d). Significant increments in DFI (%) were observed in $CuSO_4$ group (G2 group) revealed the influence of copper toxicity on sperm DNA integrity and chromatin condensation. While, rats in the L-carnitine +$CuSO_4$ treatment (G5 and G6 groups) showed a significant decrease ($p < 0.05$) in DFI signifying the protective role of L-carnitine on sperm integrity and damage (Table 3).

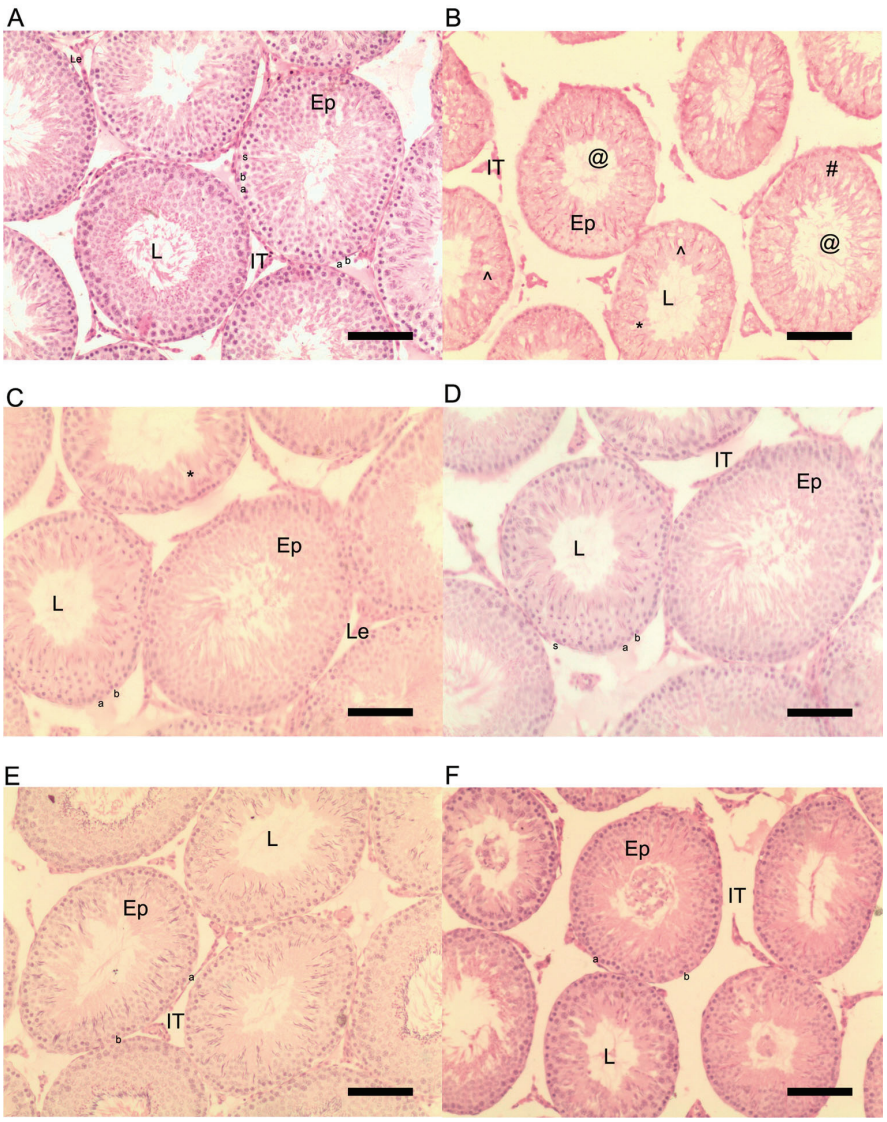

Fig. 4 Histopathological alterations in the testis of rats exposed to copper sulfate (200 mg/kg) and/or L-carnitine (50 and 100 mg/kg). **a** Control group (0.9% saline). **b** $CuSO_4$ group (200 mg/kg). **c** L-Carnitine group (50 mg/kg). **d** L-Carnitine group (100 mg/kg). **e** $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg). **f** $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg). Hematoxylin and eosin staining, ×10 eyepiece magnification, *scale bar* = 50 μm. Control **a** and L-carnitine supplemented **c** and **d** groups of animals showed normal histological characteristics while sloughing of germ cells, vacuolization, and germ cell depletion were observed in copper sulfate treated group **b**. Restoration and recuperation of seminiferous tubules damage were seen in copper sulfate plus L-carnitine treated groups **e** and **f**. Abbreviation: *a* type A spermatogonia, *b* type B spermatogonia, *Ep* seminiferous epithelium, *IT* interstitial tissue, *L* tubular lumen, *Le* Leydig's cells, *S* Sertoli cells, * accentuated depletion in the seminiferous epithelium, ^ vacuolization, @ no mature sperms in the lumen and empty lumen with cellular debris, # stages of spermatogenesis interrupted and atrophy of seminiferous tubules

Testis histopathology

Untreated control (0.9% saline) G1 group

Histopathological assessment of untreated control group G1 demonstrated all the stages of spermatogenesis with the following architecture details: (i) intact and normal seminiferous tubules (*Ep*, Fig. 4a) visibly lined with germinal epithelium; (ii) evenly distributed spermatogonia near the basement membrane in whirls; (iii) primary spermatocytes with dark stained nuclei; (iv) round and elongated spermatids after the stage of primary spermatocytes; (v) lumens of seminiferous tubules (*L*) filled with mature sperm or sperm; (vi) heads of sperms along with the spermatids and secondary spermatocytes; (vii) groups of Leydig cells (*Le*) in the interlobular spaces of seminiferous tubules; and (viii) profiles of seminiferous tubules separated by compact interstitial tissues (*IT*) were noted (Fig. 4a).

$CuSO_4$ treated (200 mg/kg) G2 group

In contrast to untreated G1 control group, the profile of seminiferous tubules in copper sulfate group G2 viewed as follows: (i) irregular with wide spaces in between them (*IT*); (ii) marked disorganization and depletion of germ cells (*); (iii) sloughing and desquamation of germinal cells (^); (iv) arrest of spermatogenesis (#); (v) splitting of spermatogonia from

Table 4 Histopathological alterations and their relative score and spermatogenic recovery found in the rat groups administered with copper sulfate and/or L-carnitine for 30 days

Categorization of seminiferous tubule damage	Control (group 1—G1)		$CuSO_4$ (200 mg/kg) (group 2—G2)		L-carnitine (50 mg/kg) (group 3—G3)		L-carnitine (100 mg/kg) (group 4—G4)		$CuSO_4$ (200 mg/kg) + L-carnitine (50 mg/kg) (group 5—G5)		$CuSO_4$ (200 mg/kg) + L-carnitine (100 mg/kg) (group 6—G6)		$F_{5,35}$ value
	Percent damage	Score	Percent damage	Score	Percent damage	Score	Percent damage	Score	Percent damage	Score	Percent damage	Score	
Types of histological alterations													
Sloughed germ cell into the lumen	15.65 ± 1.52a	1	90.65 ± 3.68b	4	13.25 ± 1.95a	1	11.71 ± 1.98a	1	18.61 ± 0.94a	1	9.88 ± 0.66a	1	239.56
Intraepithelial vacuolization	11.45 ± 1.08a	1	85.95 ± 5.66b	4	10.65 ± 1.25a	1	10.98 ± 1.42a	1	43.28 ± 1.35c	2	17.82 ± 1.76a	1	130.51
Sertoli cell-only (depletion level 1)	0a	1	51.78 ± 2.74b	3	0a	1	0a	1	10.44 ± 1.15c	1	5.10 ± 0.85c	1	258.88
Sertoli cell + spermatogonia (depletion level 2)	0a	1	73.56 ± 3.91b	3	0a	1	0a	1	8.75 ± 0.72c	1	0a	1	330.89
At least two layers of cells (depletion level 3)	0a	1	66.86 ± 2.42b	3	0a	1	0a	1	6.54 ± 0.45c	1	0a	1	715.99
Evaluation of spermatogenic recovery													
Tubule differentiation index (TDI %)	42.31 ± 3.43a		17.03 ± 2.05b		36.62 ± 1.36a		41.36 ± 1.68a		39.54 ± 1.68a		40.86 ± 1.40a		7.701

Scores 1–4, 1 = 0–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%. Score of the frequencies of the tubular alterations found in 200 tubular sections analyzed per rat ($n = 6$). Data marked with different letters (a, b) are statistically significant ($p < 0.05$) if they do not share the same letters

the intact membrane and were scant; (vi) separation and lesser number of spermatogenic cells; (vii) no mature sperms in the lumen (@); (viii) vacuolization of tubules (^); (ix) low number of Leydig's and germ cells; (x) lumen was empty or filled with debris (@); and (xi) the stages of spermatogenesis were interrupted and atrophy of seminiferous tubules (#) due to the effect of copper toxicity (Fig. 4b).

L-carnitine alone (50 and 100 mg/kg) treated G3 and G4 groups

Similar to the G1 control group, nearly normal histological features of rat testis were observed in the L-carnitine alone administered G3 group (50 mg/kg) and G4 group (100 mg/kg) wherein (i) the seminiferous tubules (*Ep*) were normal and lined with germinal epithelium, (ii) Leydig cells (*Le*) were observed in the interlobular spaces, and (iii) all sequence of spermatogenesis were present along with all types of spermatogenic cells, spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and mature sperms were present (Fig. 4c, d).

L-carnitine plus $CuSO_4$ treated G5 (50 mg + 200 mg/kg) and G6 (100 mg + 200 mg/kg) groups

L-carnitine with $CuSO_4$ treatment, group G5 (L-carnitine 50 mg/kg + $CuSO_4$ 200 mg/kg) and group G6 (L-carnitine 100 mg/kg + $CuSO_4$ 200 mg/kg), restored and recuperated the testis histomorphology and histopathological changes. After 30 days of treatment, most of seminiferous tubules had attained relatively normal structure with resumption of complete spermatogenesis in the L-carnitine treated rats (Fig. 4e, f). The arrangement of tubules was round and spermatogonia were present along with the germinal epithelium, as in the G1 control group. The severity of degenerative changes in the seminiferous tubules was less (score 1, Table 4) in G5 and G6 groups than those in the $CuSO_4$ treated G2 group (score 3 or 4) rats. Vacuolization in the seminiferous tubules was significantly decreased in the G5 and G6 groups (score 1 or 2) compared to G2 group (score 4, Table 4). Tubules exhibited regeneration in L-carnitine treated rats, whereas some of the seminiferous tubules were atrophic in the dose of 50 mg/kg L-carnitine +200 mg/kg $CuSO_4$ (Group G5). Germ cells (spermatogonia, spermatocytes, spermatids), Leydig cells, and Sertoli cells were significantly increased in number in G5 and G6 groups compared to G2 $CuSO_4$ treated group. The lumens of some tubules were empty and were in the process of refurbishment in some tubules. The germ cell population of $CuSO_4$ treated G2 group rats comprised of essentially only surviving stem spermatogonia, the most advanced cell populations detected in the repopulated tubules of L-carnitine treatment were mostly primary spermatocytes. The number of

repopulated tubules was more in G5 and G6 groups than those in the $CuSO_4$ treated G2 group rats (Fig. 4b, e, f).

Seminiferous tubule damage scoring and evaluation of spermatogenic recovery after L-carnitine supplement

Histopathological scores were computed to categorize seminiferous tubules damage due to copper treatment and to evaluate the protective role of L-carnitine supplement in the prevention of tubules damage induced by copper toxicity. The histopathological damages considered for evaluation were sloughing of germ cells, vacuolization, and three different levels of germ cell depletion (Table 4). The untreated control G1 group and L-carnitine alone treatment groups (G3 and G4) exhibited normal histological characteristics of the seminiferous epithelium and scaled the level of damage as score 1, i.e., nil germ cell depletion, 10–11% vacuolization, and < 15% sloughing of germ cells (Table 4). In the $CuSO_4$ treated G2 group animals, a vast quantity of sloughed cells (90.65%, score 4), vacuolization (85.95%, score 4), and cellular debris along with degenerative characteristics was noticed in the tubular lumen of many seminiferous tubule sections. Accentuated germ cell depletion was frequently pronounced in the seminiferous epithelium of rats from this group G2, characterizing (i) depletion level 1, which were considered severe damage (Sertoli cell-only, 51.78%, score 3); (ii) some sections showed only depletion level 2 (spermatogonia and Sertoli cells, 73.56%, score 3); and (iii) depletion level 3 (occurrence of two layers of cells, 66.86%, score 3) (Table 4). The L-carnitine (50 mg/kg) supplementation with $CuSO_4$ (200 mg/kg) treated G5 group showed lower quantity of sections with moderate damage and with moderate germ cell depletion (score 2, Table 4). While L-carnitine 100 mg/kg along with $CuSO_4$ 200 mg/kg treatment G6 group exhibiting a lower frequency of tubular sections with germ cell depletion (depletion level 1, damage—5.10%, score 1), which were considered as significantly restored group from tubule damage (Table 4). Thus, L-carnitine therapy significantly ($p < 0.05$) reduced the seminiferous tubules damage as well as protecting the tubules effectively.

The recovery of spermatogenesis was evaluated based on differentiation of tubules which contained three or more cells that had attained the type B spermatogonia (Table 4). Histological analysis of testis of $CuSO_4$ treated G2 group animals showed that $CuSO_4$ at a dose of 200 mg/kg significantly prevented the differentiation of the surviving spermatogenic stem cells in terms of tubule differentiation index (TDI %). The TDI reduced significantly in the $CuSO_4$ treatment (17.03%) whereas treatment with the L-carnitine (50 and 100 mg/kg) along with $CuSO_4$ (200 mg/kg) significantly ($p < 0.05$) restored the ability of the testis to support stem cell differentiation to 40% (intensifying the TDI) at 30 days post treatment, respectively (Table 4).

MSTD, JTBS, and quantification of germ cells and Sertoli cells

The testicular biopsy score (JTBS, 4; Fig. 5a) and seminiferous tubule diameter (MSTD, 283.75 μm; Fig. 5b) were observed with the lowest scores being evident in rats treated with a high dose of $CuSO_4$ tested. The L-carnitine alone (groups G3 and G4) and L-carnitine plus $CuSO_4$ treatment (G5 and G6 groups) showed significantly higher MSTD (292.5–318 μm, respectively) and JTBS (7.6–9.3, respectively) score when compared with those from $CuSO_4$ treated G2 group rats (Fig. 5a, b). In addition, the number of spermatogonia (Sg: 11 nos.), spermatocytes (Sc: 21.8 nos.), spermatids (Sd: 49 nos.), and Sertoli cells (St: 8.1 nos.) in the $CuSO_4$ treated G2 group rats was found to differ significantly ($p < 0.05$) than the L-carnitine alone G3 and G4 groups (Sg: 15–17.5 nos.; Sc: 25.1–28.7 nos.; Sd: 56.9–62.9 nos.; St: 9.1–10.1 nos., respectively) and L-carnitine along with $CuSO_4$ G5 and G6 groups (Sg: 21.6–25.1 nos.; Sc: 33–39.7 nos.; Sd: 53.2–63.9 nos.; St: 10.4–11.3 nos., respectively) (Fig. 5c, d). Histomorphometric analyses of copper-treated rat testes showed a significant ($p < 0.0001$) decrease in the MSTD ($F_{5,29}$ value = 5.948), JTBS ($F_{5,29}$ value = 58.473), number of spermatogonia ($F_{5,29}$ value = 33.478), spermatocytes ($F_{5,29}$ value = 12.477), spermatids ($F_{5,29}$ value = 10.989), and Sertoli cells ($F_{5,29}$ value = 5.108) which exemplify the impairment of spermatogenesis and dysfunction of testicular activity in rats by copper while these parameters restored significantly with L-carnitine supplementation showing its protective role on recovery of spermatogenesis (Fig. 5).

Immunolocalization of HSP70 protein in rat testis

Immunohistochemical staining of rat testes with an antibody directed against HSP70 revealed that HSP70 was localized in the cytoplasm of prespermatogonia (germ cells), spermatogonia, spermatocytes, spermatids, and Sertoli cells within the seminiferous tubules and Leydig cells in the interstitium (Fig. 6). The expression level of HSP70 protein in the rat testis indicated reduced expression (down regulated) in the control (Fig. 6a, G1 group), L-carnitine alone (Fig. 6c, d, G3 and G4 groups), and L-carnitine plus $CuSO_4$ (Fig. 6e, f, G5 and G6 groups) treated rats; however, overexpression (upregulated) of HSP70 protein appeared in rats treated with $CuSO_4$ at a dose of 200 mg/kg (Fig. 6b, G2 group).

Testis lipid peroxidation, antioxidant enzymes, and serum testosterone levels

The testis MDA content (a product of lipid peroxidation of the polyunsaturated fatty acid present in cell membrane) was significantly ($p < 0.05$) increased (12.21 nmol MDA/mg protein) after chronic $CuSO_4$ administration (200 mg/kg, G2 group) to rats with respect to the G1 control group (3.5 nmol MDA/mg

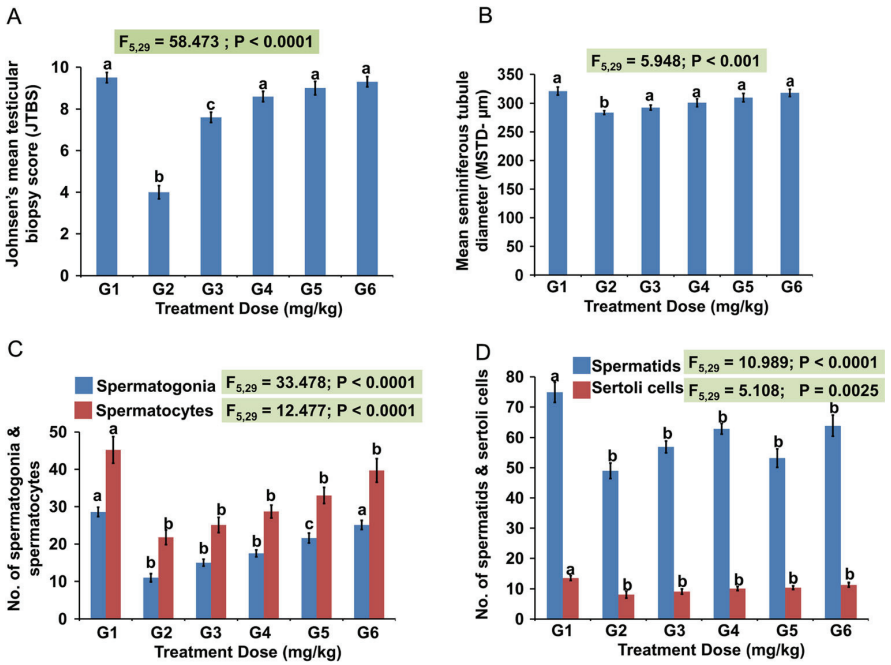

Fig. 5 Histomorphometric analysis of testis of rats exposed to copper sulfate (200 mg/kg) and/or L-carnitine (50 and 100 mg/kg). **a** Jhonsen's mean testicular biopsy score, JTBS to assess the testis damage. **b** Mean seminiferous tubule diameter (MSTD, µm). **c** Occurrence of spermatogonia and spermatocytes. **d** Incidence of spermatids and Sertoli cells. Values are mean of five observations (n = 5). All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison test. Bar with different letter means statistical significant difference at $p < 0.05$ and similar letters are not significant. G1, control group (0.9% saline); G2, $CuSO_4$ group (200 mg/kg); G3, L-carnitine group (50 mg/kg); G4, L-carnitine group (100 mg/kg); G5, $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg); G6, $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg)

protein), indicating the testicular ROS generations and induction of oxidative stress. L-carnitine alone treatment (G3 group—4.65 and G4 group—5.58 nmol MDA/mg protein) and L-carnitine cotreatment with $CuSO_4$ (G5 group—3.55 and G6 group—4.09 nmol MDA/mg protein) significantly restored these parameters toward the level of control, though a significant higher value was also noted in these parameters (Fig. 7a).

A severe inhibitory response on the testicular antioxidant status was observed, followed by chronic $CuSO_4$ exposure to rats (G2 group, Fig. 7b, c). The testicular activities of SOD (3.07 µmol/mg protein) and GST (59.28 nmol/mg protein) were significantly ($p < 0.05$) decreased in $CuSO_4$-exposed rats (G2 group) than the G1 control group (SOD, 8.55 µmol/mg protein and GST, 115.58 nmol/mg protein) signifying the suppressed testicular antioxidant defense against ROS, which assisted the generation of oxidative stress. The GST and SOD are the major antioxidant enzymes in testis tissue, which defend sperm cells by scavenging ROS generated through the exposure of environmental toxicants and pollutants. L-carnitine cosupplementation with $CuSO_4$ (G5 and G6 groups: SOD, 9.17–9.61 µmol/mg protein and GST, 117.58–118.65 nmol/mg protein) and L-carnitine alone treatment (G3 and G4 groups: SOD, 8.04–9.07 µmol/mg protein and GST, 123.58–128.65 nmol/mg protein) significantly recuperated these testicular antioxidant enzymes activities toward the control level (Fig. 7b, c).

The oral exposure of $CuSO_4$ to rats significantly decreased the serum testosterone concentrations (0.778 ng/mL, G2 group) in comparison with the G1 control rats (3.226 ng/mL), suggesting the inhibitory role of $CuSO_4$ on testicular androgenesis (Fig. 7d). However, L-carnitine alone administration (G3 and G4 groups, 3.43–3.68 ng/mL) significantly restored the serum testosterone concentrations to the G1 control group level. Moreover, significant higher values of serum testosterone concentrations were noted in the L-carnitine with $CuSO_4$ group (G5 and G6 groups, 3.32–3.74 ng/mL) when compared with the G1 control groups, suggesting that L-carnitine at a high dose of 100 mg/kg has a stronger influence on testicular androgenesis than the lower dose 50 mg/kg (Fig. 7d).

Univariate and multivariate analyses

Interactions between serum testosterone and spermatogenesis and sperm quality

A strong positive correlation was observed (1) between serum testosterone and testis damage in relation to JTBS (y = 2.825 +

1.708x; r^2 = 0.8549, Fig. A.2A) in the testis tissue; (2) between serum testosterone levels and tubule diameter (μm), MSTD (y = 278.40 + 8.585x; r^2 = 0.8622, Fig. A.2B); (3) between serum testosterone levels and sperm number in cauda ($\times 10^6$ sperms/mL) (y = 47.122 + 52.341x; r^2 = 0.9760, Fig. A.2C); and (4) between serum testosterone levels and daily sperm production in testis ($\times 10^6$ sperms/testis) (y = 9.174 + 5.060x; r^2 = 0.9455, Fig. A.2D) inferring the magnitude of copper toxicity and their influence on serum testosterone levels, spermatogenesis and sperm production, and how these physiological conditions were restored by L-carnitine supplementation.

Interactions between lipid peroxidation and serum testosterone and sperm quality

Generation of lipid peroxidation radicals (nmol MDA/mg protein) due to copper toxicity significantly disrupted the serum testosterone levels (y = 4.828–0.3211x; r^2 = 0.9093, Fig. A.3A), epididymal sperm motility (y = 76.840–0.3028x; r^2 = 0.9139, Fig. A.3B), daily sperm production (y = 13.924–0.3624x; r^2 = 0.9304, Fig. A.3C), and sperm concentration in cauda (y = 302.61–17.311x; r^2 = 0.9415, Fig. A.3D) and found with inverse relationship between the independent (lipid peroxidation) and dependent (serum testosterone, sperm motility, DSP, and sperm count) variables, respectively. While, a strong positive correlation and regression relationships were computed between MDA levels and head (y = −12.634 + 3.621x; r^2 = 0.9550) and tail (y = −7.104 + 1.742x; r^2 = 0.9559) abnormalities (Fig. A.3E, F).

MANOVA—PCA, cluster analysis, and DCA

MANOVA data matrix included of treatment groups: normal control (0.9% saline), $CuSO_4$ (200 mg/kg), L-carnitine (50 and 100 mg/kg), and L-carnitine plus $CuSO_4$ (50 and 100 mg/kg L-carnitine and $CuSO_4$ 200 mg/kg) groups as independent variables and their effect on 47 variables as dependent variables: (i) body weight; (ii) food consumption; (iii) water intake; (iv) ALT, AST, ALP, urea, and creatinine; (v) absolute and relative organ weights: liver, kidney, testes, epididymis, seminal vesicle, vas deferens; (vi) sperm count in testis, caput, and cauda and count relative to testis weight; (vii) epididymal sperm motility and viability; (viii) DSP and DSPr in testis; (ix) sperm transit time in caput and cauda; (x) total head and tail abnormalities; (xi) histomorphometrics: JTBS, MSTD, no. of spermatogonia, spermatocytes, spermatids, and Sertoli cells; (xii) percent sperm DNA damage, DFI and TDI; (xiii) serum testosterone; (xiv) oxidative stress: lipid peroxidation—MDA; and (xii) antioxidant enzymes: SOD and GST to illustrate the relationship (1) between copper toxicity and their magnitude in testis damage, suppression of spermatogenic process, impairment in sperm quality; and (2) the protective role of L-carnitine supplementation on restoration of testis damage and preservation of sperm quality (Fig. 8).

The PCA loading plot diagram indicates that the chosen 47 variables generated two principal component axes based on testicular injury by copper exposure as well as restoration activity of L-carnitine supplementation on protection of testis and sperm quality, PCA 1 accounting for 76.17% variance and Eigen value of 17,068.4 determined mainly by higher doses of L-carnitine (100 mg/kg) in $CuSO_4$ (200 mg/kg) treated rats indicating highest degree of protection of testis and sperm quality by L-carnitine supplement and PCA 2 with 17.40% variance and Eigen value of 3899.02 determined by other treatment groups. The distant and isolated variables in the plot, i.e., treatment groups, indicate a strict and extremely significant correlation between the variables (Fig. 8a). The L-carnitine plus $CuSO_4$ treatment G5 and G6 groups were separated in the PCA plot completely from the $CuSO_4$ treated G2 groups showing the modulating activity of L-carnitine against copper-induced testicular toxicity in rats and this effect is exerted by L-carnitine could be due to the broad array of biological activities including male gamete maturation, production of energy for sperm motility, DNA repair, germ cell recovery, and Sertoli cell metabolism (Fig. 8a).

The cluster analysis (Euclidean distance measure) divided the treatment groups into two major clusters according to their protective activity with the most protective group (L-carnitine + CuSO4 treatment and L-carnitine alone treatment groups) in one sub-cluster under cluster group 1, untreated control group in the second sub-cluster of cluster group 1, and no protection high risk group (CuSO4 treated rats) by the cluster group 2 (Fig. 8b). The cluster analysis clearly visualized the protective action of L-carnitine against testis dysfunction and sperm quality impairment.

The detrended analysis removes the effects of accumulating data sets from a trend and forecasting only the absolute changes in values and to allow potential cyclical patterns to be identified. The DCA and PCA analyses also detrended separated the L-carnitine supplement high dose (L-carnitine 100 mg/kg plus CuSO4 200 mg/kg) along with normal control group from the low dose L-carnitine supplement group (L-carnitine 50 mg/kg plus CuSO4 200 mg/kg) in a hierarchical fashion based on the protective action: axis 1 ranked the L-carnitine doses from more restoration of testis histology and function to less restoration groups (Fig. 8c). The PCA score plot, Euclidean distance measure, and DCA analyses distinctly showed a very good separation and there was a significant dose-dependant variation between L-carnitine supplementation and their antioxidant and protective action in comparison with the CuSO4 treated group. The results indicated that the CuSO4 exposure (200 mg/kg) group showed variation in biochemical, histopathological, immunohistochemical, and metabolic profiles than L-carnitine supplemented group rats and

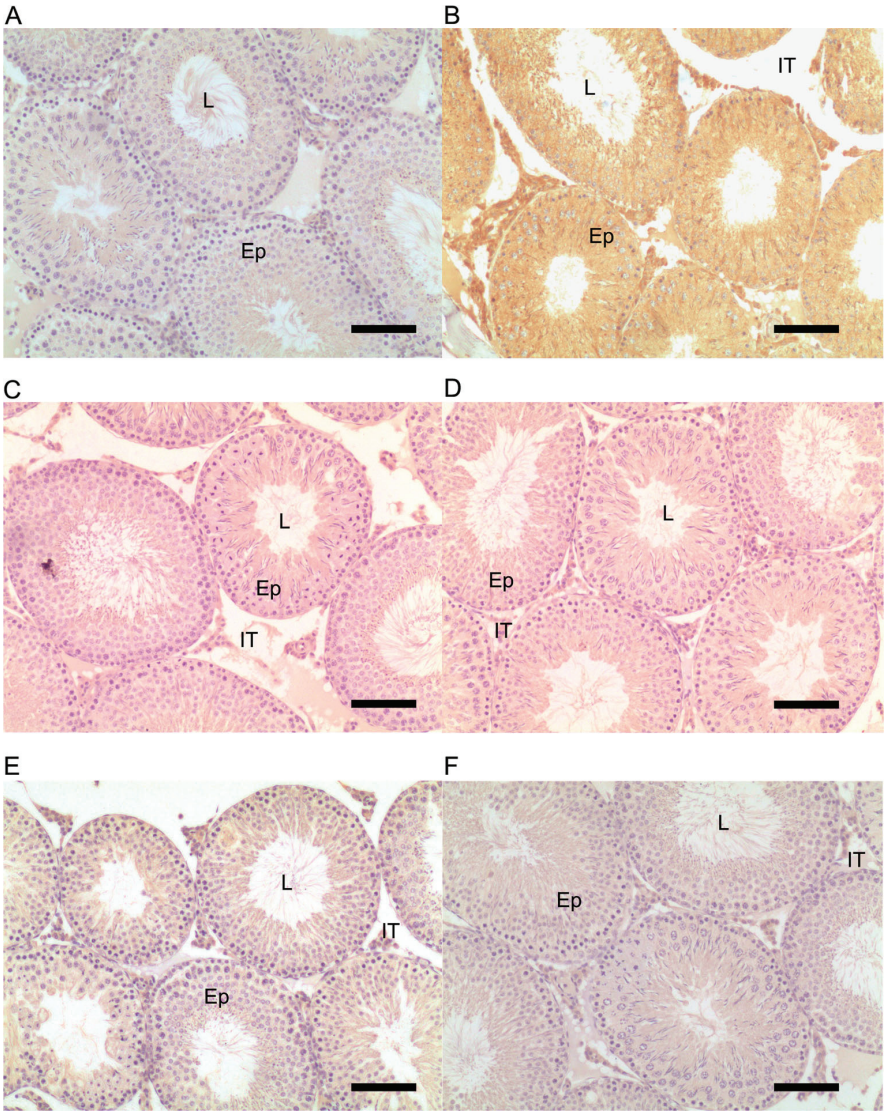

Fig. 6 Immunolocalization of HSP 70 proteins in rat testis exposed to copper sulfate (200 mg/kg) and/or L-carnitine (50 and 100 mg/kg). **a** Control group (0.9% saline). **b** $CuSO_4$ group (200 mg/kg). **c** L-Carnitine group (50 mg/kg). **d** L-carnitine group (100 mg/kg). **e** $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg). **f** $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg). Immunostaining with HSP 70 antibody, ×10 eyepiece magnification, *scale bar* = 50 μm. Down regulation of HSP 70 was observed in control **a** and carnitine supplemented **c** and **d** and copper sulfate plus L-carnitine treated groups **e** and **f** while upregulation of HSP 70 was observed in copper sulfate treated group **b**. Abbreviation: *Ep* seminiferous epithelium, *IT* interstitial tissue, *L* tubular lumen

these profiles were not affected and back to normal as a result of modulatory action of L-carnitine (Fig. 8).

The modulatory action of L-carnitine is determined by the following: (i) decreasing the testicular ROS generations and oxidative stress via inhibiting the generation of lipid peroxidation radicals (MDA), (ii) sustaining the serum testosterone levels to regulate spermatogenic process and preserving sperm quality (sperm concentration, motility, morphology, and viability), (iii) restoring the testicular function through germ cell regeneration, recovery of spermatogenic events, and retrieval of sperm DNA and chromatin integrity, (iv) regulating the antioxidant defense system for normal functioning of testis and preserving the sperm quality, and (v) inducting HSP70 proteins to perform cytoprotective role to reduce the gonadotoxicity (Fig. 9).

Discussion

Adverse effects of copper heavy metals on male reproductive health have gained much attention and can be used as a biomarker of environmental hazards. Copper testicular toxicity and its pathological features have been well corroborated in rats (Babaei et al. 2012; Babaei and Abshenas 2013). Intake of high levels of Cu adversely affected the liver and kidney which results in the rapid increase of the serum levels of ALT, AST, ALP, urea, and creatinine interpreted as an indicator of hepatic and renal dysfunction (Almansour 2006; Guclu et al. 2011). In the present study, administration of 200 mg/kg of $CuSO_4$ significantly increased serum ALT, AST, ALP, urea, and creatinine activities indicating malfunctioning of the liver

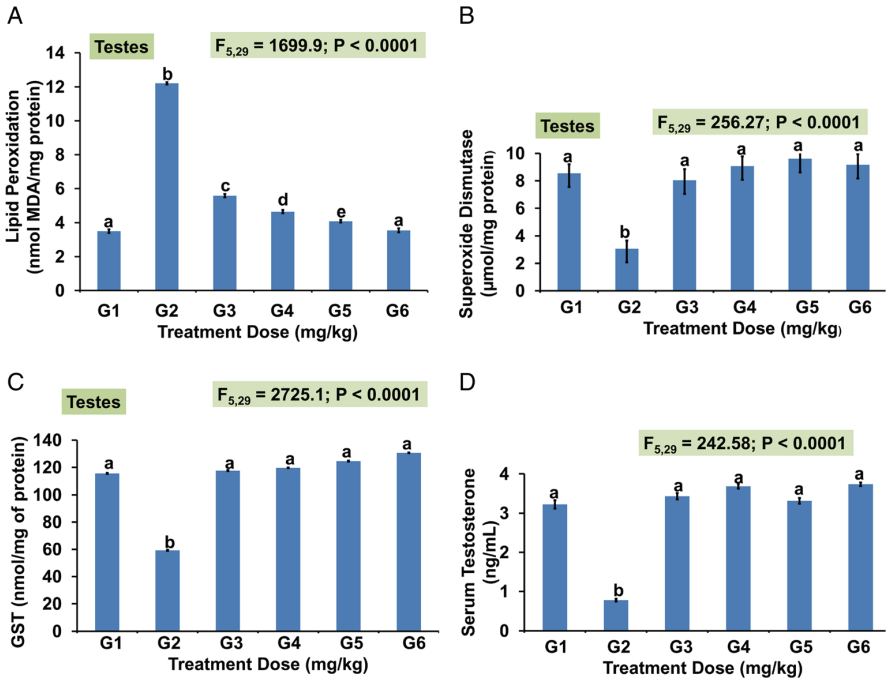

Fig. 7 Changes in serum testosterone concentrations and lipid peroxidation and antioxidant enzymes in the testis of rats treated with copper sulfate (200 mg/kg) and/or L-carnitine (50 and 100 mg/kg). **a** Assessment of generation of lipid peroxidation radicals (nmol MDA equivalents/mg protein). **b** Estimation of superoxide dismutase concentrations in the testis (SOD, µmol/mg protein). **c** Evaluation of testis glutathione-S-transferase (GST, nmol/mg protein). **d** Quantification of serum testosterone concentrations (ng/mL). Values are mean of five observations (n = 5). All values are expressed as the mean ± standard error of the mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison test. Bar with different letter means statistical significant difference at $p < 0.05$ and similar letters are not significant. G1, control group (0.9% saline); G2, $CuSO_4$ group (200 mg/kg); G3, L-carnitine group (50 mg/kg); G4, L-carnitine group (100 mg/kg); G5, $CuSO_4$ + L-carnitine group (200 mg/kg + 50 mg/kg); G6, $CuSO_4$ + L-carnitine group (200 mg/kg + 100 mg/kg)

and kidney while L-carnitine supplemented rat groups did not reveal any significant alteration in the activities of these biochemical parameters indicating the modulator effect of L-carnitine.

Malfunctioned endocrine system both at hypophysis and testicular levels is competent enough to adversely influence the spermatogenesis. As a result, the luteinizing and follicle-stimulating hormones stimulate the testicular endocrine activities (Akkoyunlu et al. 2007). Consequently for impaired endocrine interactions, testicular tissue experience to ROS and reactive nitrogen species (RNS)-dependent stresses (Agarwal et al. 2008). Testosterone is found to be indispensable for normal spermatogenesis as it promotes the switching of round spermatids into elongated spermatids between stage VII and stage VIII of the spermatogenetic cycle. Androgen insufficiency perturbs the spermiation process (Saito et al. 2000) by shifting spermatid-Sertoli cell junctions, which outcomes in premature detachment of round spermatids from Sertoli cells and seminal epithelium (Beardsley and O'Donnell 2003), along with apoptosis and activation of caspases (Tesarik et al. 2002). In this study, the observation that chronic ingestion of $CuSO_4$ significantly reduced serum testosterone levels in group 2 was in accordance with the aforementioned findings. This decrease in testosterone levels led to a decrease in spermatogenesis. In this line, the correlation between generation of ROS/RNS and infertility has been documented well. Hammami et al. (2009) hypothesized that steroidogenesis is inhibited in three different ways: (i) disorder in free cholesterol mobilization toward Leydig cell mitochondria, (ii) interruption of cholesterol mitochondria translocation with the steroidogenic acute regulatory (STAR) protein as an effector, and (iii) prevention of cholesterol conversion into testosterone by impairing activities of key regulatory enzymes of steroidogenesis. Possibly the reduction in serum testosterone level observed in this study may have been caused by one of these hypothesized mechanisms, but this is the issue of future research.

L-carnitine has antioxidative, antiperoxidative, and free radical scavenging properties which protect the cellular DNA and membranes against cellular damage induced by free oxygen radicals (Ferrari et al. 1988; Izgut-Uysal et al. 2003). L-carnitine protects the germ cells from oxidative stress by preventing the formation of ROS produced by the xanthine/xanthine oxidase system and decreases damage to the cell membrane (Bertelli et al. 1994). The assessment of sperm chromatin integrity along with the sperm head and tail

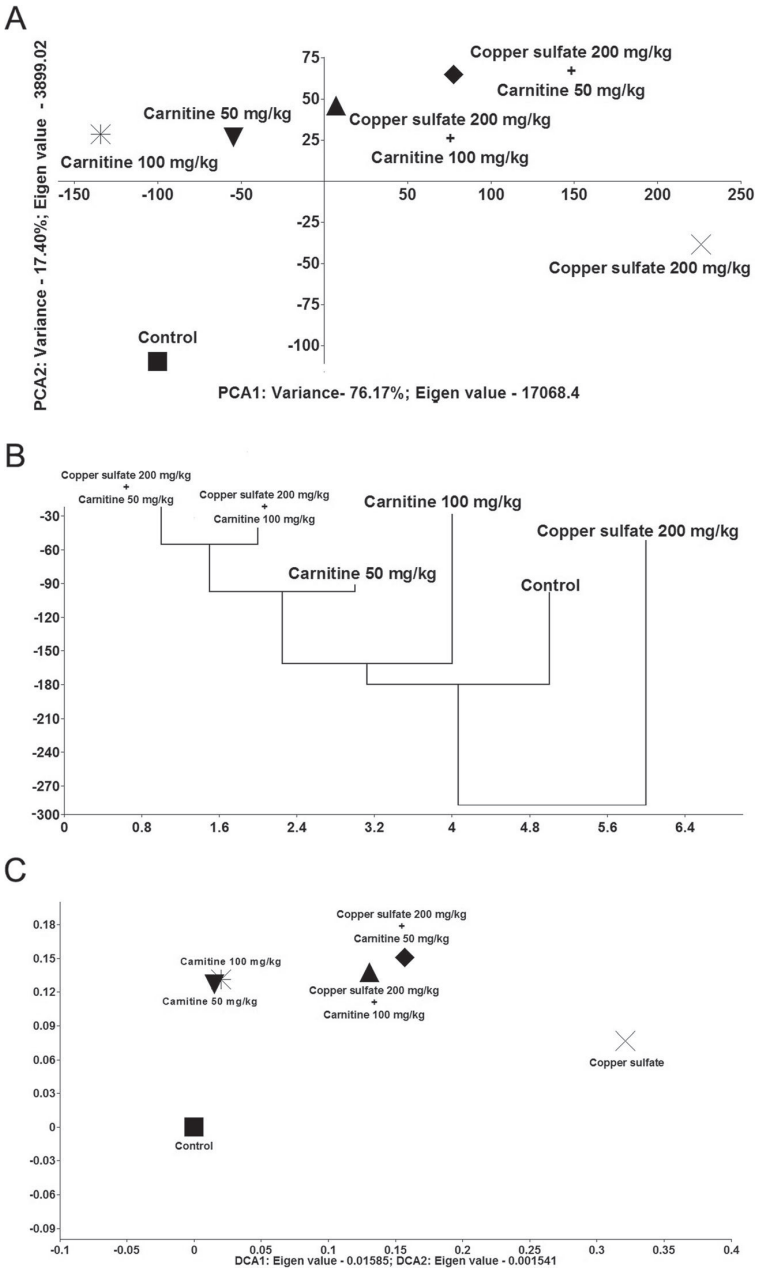

Fig. 8 Assessment of testicular toxic nature of copper and protective action of L-carnitine through multivariate analysis of variance (MANOVA). **a** Principal component analysis (PCA) indicating of copper toxicity and their degree of response and ameliorative action of L-carnitine supplementation in exhibiting antioxidant and anti-inflammatory properties. MANOVA data matrix consists of six treatment groups as independent variables and 47 dependent variables (refer test) to exemplify the association between the toxic effect of copper on testis and gonadoprotective action of L-carnitine. **b** Euclidean cluster analysis categorizes the treatment groups based on magnitude of copper toxicity and gonadoprotective action of L-carnitine. **c** Detrended correspondence analysis (DCA) separates the treatment groups in a hierarchical fashion reflecting the gonadotoxic nature of copper and restorative action of L-carnitine. Loadings plot reveal the variables responsible for the discrimination of the treatment groups

morphological alterations is important for ascertaining and scoring germ cell toxicants and the generation of single and double strand breaks in the sperm DNA is characteristically associated with genotoxic agents, including heavy metals (Trivedi et al. 2010). In our observation, exposure of $CuSO_4$ significantly elevated the lipid peroxidation (a biomarker of oxidative stress), impaired the antioxidant defense system by reducing the antioxidant enzymes (SOD and GST), suppressed the germ cell proliferation and damaged the DNA chromatin integrity by increasing sperm DNA damage and decreasing DFI (more AO^+, yellow/red fluorescent sperms, single strand breaks). Thus, it was confirmed that copper is toxic to germ cells and acts as a genotoxic agent. Conversely, these parameters were significantly restored and regulated by L-carnitine supplementation inferring its modulatory action.

Besides, L-carnitine participates in sperm cell metabolism and supply energy for sperms, which significantly influences maturation and motility of sperms and the spermatogenic

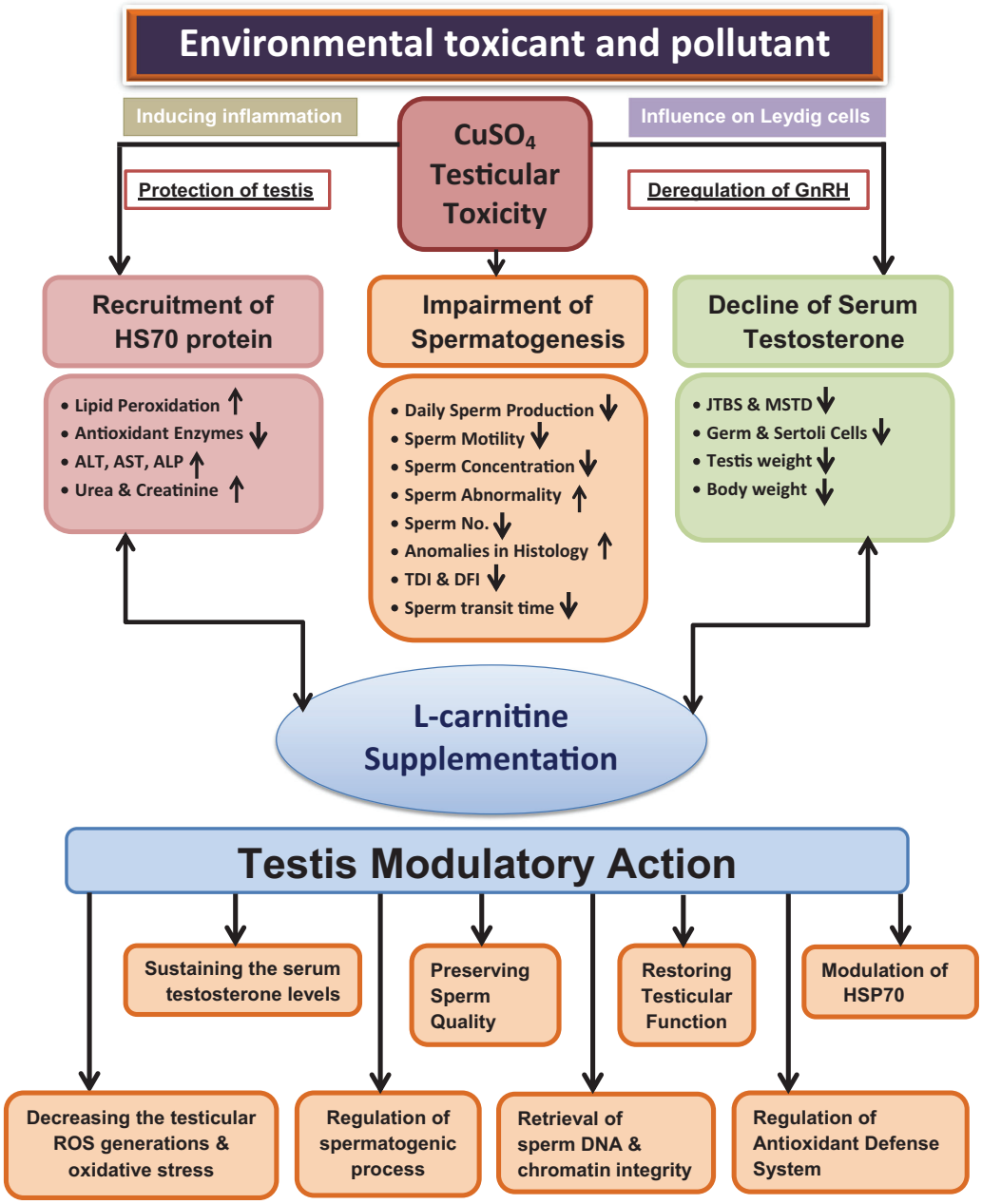

Fig. 9 Modulatory action of L-carnitine against copper-induced testicular toxicity in adult Wistar albino rats

process (Matalliotakis et al. 2000; Ruiz-Pesini et al. 2001). These findings are in corroboration with our study outcomes in L-carnitine supplemented rat groups as: (1) revitalization of sperm quality (motility, viability, number, DSP, and DSPr), (2) restoration of histological alterations (germ cell depletion, sloughing of germ cells, vacuolization, and degeneration), and (3) rejuvenation of spermatogenesis in terms of increase in JTBS, MSTD, TDI, and number of germ cells (spermatocytes, spermatogonia, spermatids, Sertoli cells, Leydig's cells) than $CuSO_4$ treatment group. Morphological abnormality of sperm (head and tail abnormalities) is the characteristic feature that persuade with weak sperm motility (Roychoudhury et al. 2008; Slivkova et al. 2009; Pekala et al. 2011). Several metabolic and biochemical profiles interfered with spermatogenesis and reduce sperm quality and production in Wistar rats. The abnormal sperm, damaged germinal epithelium, and infiltration

of immune cells are the consequences of generation of elevated amounts of ROS in the testis (Forlenza and Miller 2006). Conversely, the increase of ROS are accountable for oxidating the NO into peroxynitrate, which is causing gonadotoxicity (Agarwal et al. 2008). Moreover, the high generation of ROS/RNS results in significant damages in sperm DNA integrity, motility, and plasma membrane fluidity (Rezazadeh-Reyhania et al. 2015). Exposure of copper to rats significantly increased the lipid peroxidation radicals which in turn affecting sperm DNA quality, yielding sperm head and tail abnormalities, and finally upsetting the sperm quality. While these metabolic and physiological conditions were revert back significantly by L-carnitine treatment. Optimal sperm transit time (8–15 days) through epididymis is required for sperm to attain maturation and motility which is vital for sperms to fertilize ova (Bellentani et al. 2011). The sperm transit time in caput and cauda was accelerated in $CuSO_4$ treated rats which may decrease the sperm reserves in the caudal portion of epididymis and interfere with the reproductive process of rats. This condition was revived with L-carnitine coadministration. HSP70 proteins have an important role in the male testis as trafficking and translocating proteins through membranes during stress conditions to protect the cellular functions. It can be used as a biomarker to assess the oxidative stress (Biggiogera et al. 1996; Wu et al. 2011). Localization of HSP70 was upregulated in $CuSO_4$ treated rat testis to protect the germ cell proliferation as well as to maintain the testis function while it was downregulated in L-carnitine supplemented rat testis. Hence, HSP70 can be used as an oxidative stress marker for testicular toxicity.

Conclusions

$CuSO_4$ at a dose of 200 mg/kg severely affected the spermatogenesis (histological alterations), testis function and caused testicular toxicity. The body and organs weights, food consumption, water intake, serum biochemical profiles (ALT, AST, ALP, urea, and creatinine), serum testosterone levels, and antioxidant enzymes (SOD and GST) were significantly disturbed and altered. Sperm quality (motility, viability, concentration, DSP, DSPr, DNA integrity, and DFI) was significantly affected and observed defective sperms with head and tail abnormalities. The sperm transit time in caput and cauda was accelerated and resulted in incomplete spermatogenic process and formation of immature sperm leading to infertility. L-carnitine supplementation significantly improved the physiological and metabolic processes and in turn recovered the spermatogenic process, germ cell proliferation, DNA repair, and sperm quality. Based on these findings, L-carnitine could be used as a therapeutic as well as protective agent for copper heavy metal testicular toxicity in rats.

Acknowledgements The authors acknowledge the instrumentation facility in Mizoram University funded by Department of Biotechnology, Government of India, New Delhi—Bioinformatics Infrastructure Facility (No. BT/BI/12/060/2012(NERBIF-MUA) and State Biotech Hub Programme (No. BT/04/NE/2009).

Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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*Isolation, characterization, and therapeutic activity of bergenin from marlberry (*Ardisia colorata *Roxb.) leaf on diabetic testicular complications in Wistar albino rats*

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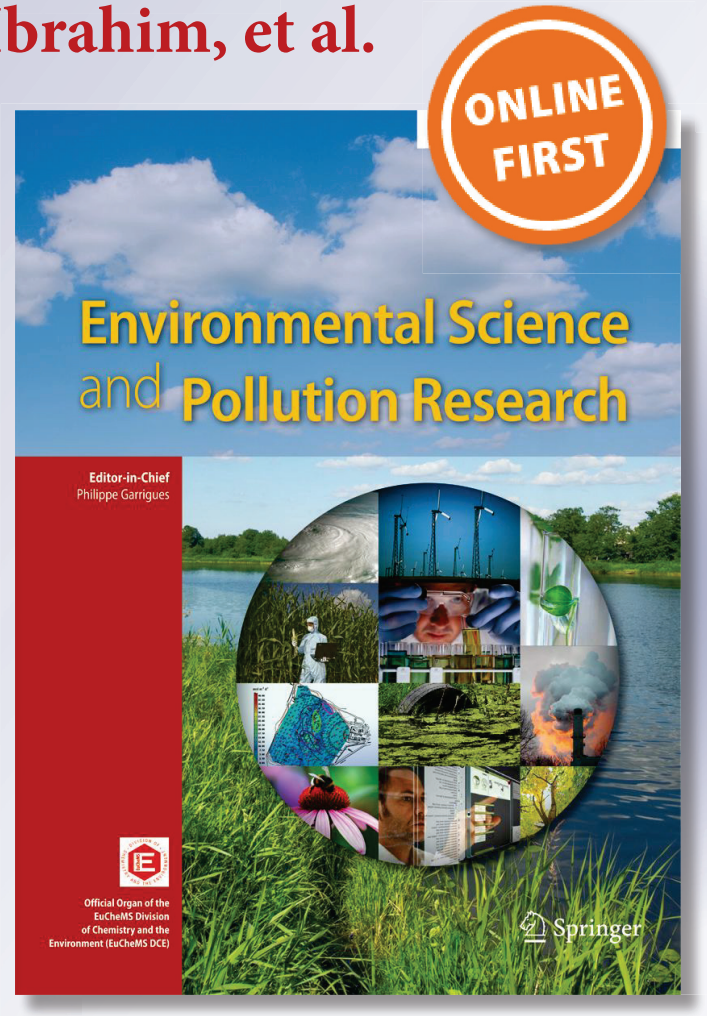
Environmental Science and Pollution Research

ISSN 0944-1344

Environ Sci Pollut Res
DOI 10.1007/s11356-019-04139-9



Springer

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-019-04139-9

RESEARCH ARTICLE

Isolation, characterization, and therapeutic activity of bergenin from marlberry (*Ardisia colorata* Roxb.) leaf on diabetic testicular complications in Wistar albino rats

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Received: 17 September 2018 / Accepted: 2 January 2019

Abstract

Bergenin is one of the phytochemical constituents in marlberry (*Ardisia colorata* Roxb.) having antioxidant, anti-diabetic, and anti-inflammatory properties. *A. colorata* has been used as an herbal medicine in Southeast Asia particularly in Northeast India to treat diabetes. Bergenin was isolated from methanol extract of *A. colorata* leaf (MEACL) by column chromatography and TLC profiling. Characterization and structural validation of bergenin were performed by spectroscopic analyses. A LC-ESI-MS/MS method was developed for the quantitation of bergenin and validated as per the guidelines of FDA and EMA. The validated method was successfully utilized to quantify bergenin concentration in MEACL samples. Therapeutic efficacy of bergenin was investigated on streptozotocin-induced diabetic rats by following standard protocols. Bergenin supplementation significantly improved the physiological and metabolic processes and in turn reverses diabetic testicular dysfunction via increasing serum testosterone concentrations and expression pattern of PCNA, improving histopathological and histomorphometric manifestations, modulating spermatogenic events and germ cell proliferation, restoring sperm quality, reducing sperm DNA damage, and balancing the antioxidant enzymes levels. Hence, *A. colorata* leaf is one of the alternate rich resources of bergenin and could be used as a therapeutic agent for diabetic testicular complications.

Keywords Coral berry · Trihydroxybenzoic acid glycoside · Structure identification · Assay validation · Hyperglycemia-induced testis dysfunction · Sperm quality

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-019-04139-9) contains supplementary material, which is available to authorized users.

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Published online: 15 January 2019

Science and Technology Journal Vol. 6 Issue 1 January 2018 ISSN: 2321-3388

Cadmium Toxicity and Its Effect on Testicular Dysfunction in Male Rats

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Abstract—Cadmium (Cd) is an inorganic heavy metal which is of significant occupational concern, has been classified as human carcinogen. Administration of Cd in animals results in multiple organ tumorigenesis. Evidences indicate that the oxidative stress plays an important role in Cd carcinogenesis. Cd exposure has been related to human diseases such as cancer and studies have shown the adverse effects of different doses of cadmium chloride ($CdCl_2$) on reproductive functions in male. Studies have shown that $CdCl_2$-induced testicular and spermatozoa toxicity associated with the oxidative stress in male downregulating the apoptotic genes and up regulating gonadotropins and anti-apoptotic genes. $CdCl_2$ toxicity significantly decreases the reproductive organ weights, epididymal sperm concentration, motility and B-cell lymphoma 2 (*BCL 2*) positive anti-apoptotic cell rate, whereas it caused significant increases in level of lipid peroxidation and abnormal sperm rates. *BAX* gene expression has been detected in leydig cells, indicating that *BAX* gene is a promotor of cell death is often positively regulated by the tumour suppressor gene p53 and may also cause the formation of multinucleated giant cells which is caused by additional DNA replication of the primary spermatocytes that fail to undergo meiosis. Thus, this article reviews some of the molecular toxicity mechanism of Cd and its effect on the testis which will be helpful in anticipation and deterrence of Cd-induced testicular toxicity.

Keywords: Cadmium Chloride, Exposure, Humans, Testis, Apoptosis, Oxidative stress

INTRODUCTION

Cadmium is a commonly used chemical in agricultural and industrial operations and is released into the environment causing atmosphere, soil, and water pollution. Cadmium can accumulate in the body for up to 40 years, so it can harm human health over an extended period of time (Wang *et al.* 2016). The general population is exposed to Cd via contaminants found in drinking water and food (ATSDR, 2007). There has been a surge in Cd pollution of the environment partly due to urbanization resulting in increase in Cd exposure in human beings which is subsequently leading to higher incidence of Cd-related diseases. Administration of Cd significantly increased the relative weight of testis of rats (Wang *et al.* 2016; Adaramoye *et al.* 2016). Lipid peroxidation was significantly increased while antioxidant parameters decreased in testis of Cd-treated rats. Also, Cd-treated rats had significantly reduced sperm count, motility, sialic acid, luteinising hormone and testosterone relative to controls (Adaramoye *et al.* 2016).

Cadmium is a toxic heavy metal, which probably cause infertility by impairment in spermatogenesis (Yari *et al.* 2016). With high levels of exposure to toxic chemicals including heavy metals, released into the environment and introduced in the food chain, the health of living organisms is affected, sometimes with fatal consequences (Predes *et al.* 2016). In fact, the International Agency for Research on Cancer classified Cd as a known human carcinogen in 1993 (IARC, 1993) and Cd is ranked the 7th toxicant in the Priority List of Hazardous Substances (Priority List) of the Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR, 2007). Recent studies have demonstrated that the testis is sensitive to cadmium, but studies investigating cadmium-induced testicular injury have not yet clearly revealed the underlying mechanisms. Hematoxylin and

http://doi.org/10.22232/stj.2018.06.01.08

Theriogenology 88 (2017) 73–83

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Biosterilant effects of *Bacillus thuringiensis kurstaki* HD-73 extract on male Wistar albino rats

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ARTICLE INFO

Article history:
Received 21 April 2016
Received in revised form 1 October 2016
Accepted 3 October 2016

Keywords:
Bacillus thuringiensis
Biosterilization
Wistar albino rat
Histopathology
Antioxidant enzymes
Azoospermia

ABSTRACT

Chemosterilants have long been used in sterilization programs for managing pet, stray, and wild animals but adverse effects such as trauma, incomplete responses to treatment, and complete abolition of gonadal sources of testosterone often occurs. This study describes the biosterilant effects of administering three doses of *Bacillus thuringiensis kurstaki* HD-73 (Bt 5, 50, and 250 mg/kg; 20 rats/dose) extract in adult male Wistar albino rats on testicular parameters, function, histology, and a number of biochemical markers of overall health, free radical production, and cell proliferation. Intratesticular administration of Bt extract to rats induces testicular oxidative stress and damages and consequently, perturb spermatogenesis, degeneration of testis, reduction in testes size, and depletion of testosterone and antioxidant enzyme concentrations in a dose-dependent manner because of free radical–mediated lipid peroxidation. No morbidity or mortality adverse effects were observed in both the saline control and Bt extract–treated rats. Significant variation was noted in clinical manifestations, weight and volume of testes, and hormonal and biochemical profiles between Bt doses in comparison with the saline control. Aspermia/azoospermia (100%) resulted in Bt-treated rats without any adverse effects. Histopathological analysis showed degeneration, necrosis, vacuolation, fewer germ cells, formation of multinucleated giant cells, and a lack of elongated spermatids in atrophic seminiferous tubules in Bt extract–treated groups in the presence of low concentrations of testosterone, antioxidant enzymes, and suppression of germ cell proliferation. Dose-dependent effects were evident in most parameters that were measured. The vast array of tests that were undertaken also provides some important indicators of the physiological effects associated with the treatments that were applied. Intratesticular injection of Bt extract impairs spermatogenesis and induces permanent sterility in rats.

1. Introduction

Nonsurgical form of contraception using chemicals (formalin, ethanol, cadmium chloride, ferric chloride, ferrous sulfate, glycerol, calcium chloride, zinc gluconate, and sodium chloride) has long been practiced as a promising and most effective means of managing the domestic and wild animals [1–10]. It is an inexpensive, affordable, and suitable method for large-scale sterilization programs and further chemical sterilants used in this process would be permanent, delivered in a single treatment and arrest androgenesis, libido, and spermatogenesis [1,4,11,12]. Disadvantages associated with many of these chemosterilants include incomplete and inflammatory responses to

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0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.10.004

Science and Technology Journal, Vol. 5 Issue: 1 ISSN: 2321-3388

Diosgenin: Therapeutic Action, Pharmacology and Applications

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Abstract—Diosgenin [25R-spriost-5-en-3β-ol], is an important steroidal metabolite found in various plant species. The discovery of diosgenin has made it one of the most researched and studied herbal product. Moreover, there is excellent opportunity to address whether diosgenin plays a role in chemoprevention versus therapy, or both. However, rigorous experimental based evidence in support of ethnomedicine-derived notions would lead to the development of products relevant to drug development. The health beneficial effects of diosgenin are further extended to its potential role to treat other ailments such as HIV and hepatitis-C infections as well as liver diseases. There is little information regarding the bioavailability, pharmacokinetics and pharmacodynamics of diosgenin in relation to its health beneficial effects. It has been reported to have wide spectrum of biological properties that contributes to several diseases in its role as a health beneficial phytochemical by citing new studies.

INTRODUCTION

Diosgenin, a bioactive steroidal saponin from legumes and yams, (Chen *et al.* 2015; Fuller *et al.* 2015; Miyoshi *et al.* 2011; Raju and Mehta (2009) is an anti-inflammatory, antidiabetic, antitumor, immunomodulatory, neuroprotective, estrogenic, vasodilatory compound, skin protective potency which also reduces blood lipid content and protects against ischemia induced neuronal damage (Cailleteau *et al.* 2008; Chen *et al.* 2011; Chen *et al.* 2015; Esfandiarei *et al.* 2011; Gong *et al.* 2010; Lepage *et al.* 2011; Roghani-Dehkordi *et al.* 2015; Li *et al.* 2010; Li *et al.* 2015) However, a limited number of studies have been performed on the reproductive effect of diosgenin on female; the study will elucidates the ability and molecular mechanisms of diosgenin. This review will provide the beneficial role of diosgenin against metabolic diseases and pharmacological effects (Fig. 1).

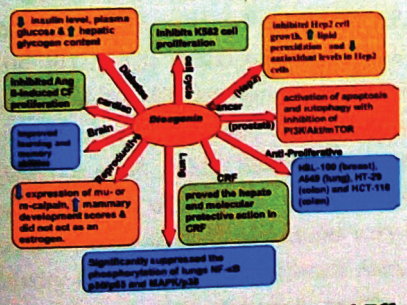

Fig. 1: Diosgenin and its Pharmacological Effects (Kanika *et al.*, 2012)

http://doi.org/10.22232/stj.2016.04.02.07

BIOLOGICAL ACTIVITIES OF DIOSGENIN

Diosgenin, a steroid saponin, is a major bioactive constituent of various edible pulses and roots, found in plants such as *Dioscorea bulbifera, Dioscorea alata, Dioscorea trinervia. Dioscorea deltoidea, Dioscorea oppositifolia, Solanum xanthocarpum* as well as in the seeds of fenugreek (*Trigonella foenum graecum*) (Kanika *et al.* 2012). In the pharmaceutical industry, diosgenin is a well-known precursor in the manufacture of synthetic steroidal drugs (Raju and Mehta 2009). Diosgenin possess a wide range of health beneficial properties, which has been used in traditional medicine as an antihypercholesterolemia, antihyperglycemia and antidiabetes agent. Over the past decade, a series of preclinical and mechanistic studies have showed that diosgenin could inhibit proliferation and induce apoptosis in a wide variety of tumor cells, including osteosarcoma, colon carcinoma, leukemia, hepatoma and breast carcinoma (Chiang *et al.* 2007; Li *et al.* 2010; Moalic *et al.* 2001). The anti-tumor mechanisms of diosgenin has been demonstrated via modulation of multiple cell signaling events involving critical molecular candidates associated with growth, differentiation, apoptosis and oncogenesis (Chen *et al.* 2011). Therefore, these preclinical and mechanistic findings strongly implicate that diosgenin may possess the cancer chemotherapeutic potential as a novel, multi target-based therapeutic agent against many cancer types (Fig. 2 and 3).

Science and Technology Journal, Vol. 5 Issue: 1 ISSN: 2321-3388

Implications of Use of Tobacco Smoke Infused Water

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Abstract—Smokeless Tobacco Products have been collectivelydesignated as Group 1 carcinogens i.e. carcinogenicto humans.It is estimated that the use of tobacco kills about three million people globally every year. A number of smoking and smokeless tobacco products are in use all over the world. Unlike other smokeless tobacco products, a unique water (liquid preparation) containing the extracts of tobacco smoke is used in Mizoram and is locally known as *tuibur (tobacco smoke infused water)*.This product is made locally by passing smoke, generated by burning tobacco, through water till the preparation turns cognac in colour and has a pungent smell.

Keywords: Tobacco, Tuibur, Toxicity

INTRODUCTION

SMOKELESS TOBACCO

Smokeless tobacco (chewing or spit tobacco) epidemic has long been recognized as a global threat and a challenge to human health affecting>300 million people worldwide (Biswas *et al.* 2015). South Asia is a global hub for the production and supply of Smokeless tobacco (> 33%) to the remaining part of the world and the consumers of the smokeless tobacco in Southeast Asia is about 90% of the tobacco usersworldwide (Sinha *et al.* 2015). The main producer consisting huge number of consumer and exporter of tobacco is South Asia. Although India being the second largest consumer of tobacco (274.9 million), use of smokeless tobacco surpasses prevalence of smoking (smokeless tobacco products: 163.7 million; smoking: 68.9 million; smokeless tobacco and smoking: 42.3 million) (Global *et al.* 2015). The practice of smokeless tobacco is culturally accepted in India (current users: 25.9% of adults) and is prevalent both in men (1.3–38%) and women (4.6 - 27.9%) (Giovino *et al.* 2015). Several smokeless tobacco products are available worldwide, manufactured through different processes and their predilections vary by country, region, ethnicity and gender. In Southeast Asia, smokeless tobacco products are manufactured mostly small and cottage industries and their use are connected with lower socioeconomic status and rural residence (Kyaing *et al.* 2012). Smokeless tobacco product are widely consumed in India and partly across the global population which contains many toxicants (nicotine, polycyclic aromatic hydrocarbons, nitrate, nitrite, acrolein, crotonaldehyde and metals) and twenty eight known carcinogenic complexes in which tobacco specific nitrosamines are believed to be the most potent one (Bhisey *et al.* 2012).

Use of smokeless tobacco (snuff, tobacco powder for oral use, gutka, etc.) or cigarette smoking is known to cause various forms of cancers as various chemical constituents of tobacco are found to be carcinogenic. A carcinogen is defined as a chemical, physical or biological agent that causes cancer or induces the incidence of cancer. Even then, consumption of tobaccois the principalcaused of global preventable death, it is estimated that the use of tobacco in diverse forms leading to 6 million people deaths globally every year (WHO 2012) with a majority occurring in Asia, and this figure is expected to rise further. Smokeless tobacco use is very high, among female users, in North-eastern India (Galażyn-Sidorczuk *et al.* 2008; Kalicanin *et al.* 2012). Although, the annual death figure due to tobacco consumption increases every year, cessation of tobacco consumption will not happen anytime in near future. And, if this trendincreases, tobacco caused mortalities will reach 8 million worldwide annually by the year 2030 (Malakar *et al.* 2012).

http://doi.org/10.22232/stj.2016.04.02.07

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NAME OF CANDIDATE	:	KHUSHBOO MAURYA
DEGREE	:	DOCTOR OF PHILOSOPHY
DEPARTMENT	:	ZOOLOGY
TITLE OF THESIS	:	ROLE OF DIOSGENIN ON SPERMATOGENESIS AND STEROIDOGENESIS IN SWISS ALBINO MICE
DATE OF ADMISSION	:	04.08.2014
APPROVAL OF RESEARCH PROPOSAL	:	
(i) Board of Study	:	15.05.2015
(ii) Board of School	:	22.05.2015
REGISTRATION NO. & DATE	:	MZU/Ph.D./726 of 22.05.2015
EXTENSION (IF ANY)	:	-

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ABSTRACT

ROLE OF DIOSGENIN ON SPERMATOGENESIS AND STEROIDOGENESIS IN SWISS ALBINO MICE

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ABSTRACT

Introduction

Diosgenin, a major steroid reported in fenugreek, *Costus* and *Dioscorea* species are known for its lowering blood sugar and lipid content in case of hyperglycemia (McAnuff*et al.*, 2005) and hyperlipidemia (Al-Matubsi *et al.*, 2011) respectively. Recently, its therapeutic and pharmacological activity has received a lot of attention in various pathological conditions such as disorder in metabolism of sugar and fat and related organ damage difficulty. Diosgenin has been widely used in industrial synthetic production of sex steroids like corticoids, progesterone, testosterone and estrogen (Chiang *et al.*, 2007). However due to its strong antioxidant effect against inflammations and free radical species, diosgenin has received attention towards its pharmacological effect on various kind of cancer and arthritis (Ma *et al.*, 2011). In an experimentally induced diabetic rat model, diosgenin enhanced glucose homeostasis in blood via improving hepatic enzyme function (McAnuff*et al.*, 2005b). Moreover, diosgenin has been used for estrogen deficiency and bone loss during female menopausal condition (Chiang *et al.*, 2011). Recently, diosgenin has reported as anti-depressive (Ho *et al*, 2012) and anti-ageing drug (Ho *et al*, 2012). Beside of that, diosgenin has an important role in glucose and fat metabolism (Son *et al.*, 2007; McAnuff *et al.*, 2005). Several other function have been reported like anticancer effects in proliferating cancerous cell (Das *et al.*, 2012), anti-bone loss in estrogen deficiency (Chiang *et al.*, 2011), anti – depressive (Ho *et al.*, 2012), anti- osteoporosis (Chen *et al.*, 2018) and anti-ageing effect (Ho *et al.*, 2012). Most interestingly, several researchers reported diosgenin as estrogenic agent

(Aradhana *et al.*, 1992; Accatino *et al.*, 1998) because its structure shows similarity with estrogen as well as progesterone (Higdon *et al.*, 2001; Au *et al.*, 2004). Furthermore, diosgenin, being a precursor of several synthetic steroid hormone productions, is well known option for menopausal (premenopausal and postmenopausal) disorder in aged women. In case of male aging disorder, diosgenin administration in aging rat model improves volume and density of bone as in case of osteoporosis (Ho *et al.*, 2014). Most recent, diosgenin exposure reveals the altered sexual dysfunction by D-galactose induced aging (Yu *et al.*, 2011).

Researchers have paid great attention to steroid like compounds present in numerous dietary agents because of associated reproductive risks due to the side effect of herbal plants. While good deals of research have been conducted on the reproductive toxicity of these plants, there is a lack of information about the mechanisms involved in the pathogenesis of male reproductive dysfunction induced by diosgenin. Based on the previous and recent literature data, we hypothesized that the diosgenin, a potent new synthetic steroid precursor agent, widely used in pharmacological and industrial purpose; may interfere in testicular function in normal healthy mice. Interestingly, researched done by Medigovi´c *et al.* (2014), revealed diosgenin does not act as estrogenic agent in immature rat model of an uterotrophic assay, has opened a new way of our research in male reproductive system. Although, estrogenic activity of diosgenin has been already well reported but there is a lot of confusion and doubts about its effect on gonads and reproduction. The present study highlighted the effect of diosgenin on sperm formation and testicular steroidogenesis.

Objectives:

This work aims to evaluate and compare the effects of diosgenin on the spermatogenesis and steroidogenesis in mice:

- Oxidative stress and antioxidant defence activities;
- Sperm and serum hormone analyses; and
- Histopathological and immunohistochemical changes in testes.

Materials and methods:

Animal maintenance and experimental design

All the experiment was conducted following the guidelines approved by the Mizoram University Institutional Animal Ethics Committee of Mizoram University, Mizoram, India (**MZU permit number: MZU/IAEC/18/12**). This work was approved by both committee for the purpose of "Control and Supervision on Experiment on Animal" and "Ministry of Environment and Forests" New Delhi, India. For testing of acute oral toxicity of chemicals, acute toxicity study was conducted as per the OECD guidelines (OECD Guidelines for the Testing of Chemicals. 2001) To estimate the LD_{50} of diosgenin exposure, the method described by Hamilton and Attia, (1978) has been adopted.

Twenty five male Swiss albino mice, 56 days old, weighing 25 to 30 g, were selected for this study. Maximum of 5 animals in each cages were housed in sterilized plastic polypropylene cages well covered with stainless steel grill in a

controlled-environment animal room (temperature, 23 ± 1 ºC; relative humidity, 50 ± 10%; photoperiod, 12 h light/dark cycle). There was free access for drinking water and sterilized food (ad libitum) allowed at all through the time. Diosgenin treatments were administered for 40 days (one spermatogenic cycle). All diosgenin treated group received diosgenin in vehicle solution (1 mL 20% twin twenty in 100 mL of 70% ethanol).

All selected animals were allowed to acclimatize for the inspection and quarantine for 7 days prior to treatment begins.

Table 1. Experimental design for diosgenin treatment

Groups	Dose	No. of animals
Group- 1	**Control (Normal control)**	**N = 05**
Group- 2	**10 mg/kg/b.w Diosgenin**	**N = 05**
Group- 3	**50 mg/kg/b.w Diosgenin**	**N = 05**
Group-4	**100 mg/kg/b.w Diosgenin**	**N = 05**
Group-5	**200 mg/kg/b.w Diosgenin**	**N = 05**

Test chemicals

Diosgenin in the form of pure powder, purchased from Sigma Aldrich ($C_{11}H_{10}N_2O_3$, molecular weight 218.21 g/mol, CAS No: 60875-16-3, purity 98%) was obtained from Beijing Zhongnongfa Pharmaceutical Co., Ltd. (Huanggang, China).

Testicular oxidative stress and antioxidant defence activity

Testis tissue was used to evaluate the level of malondialdehyde (MDA, nmol mg-1 protein) by measuring the thiobarbituric acid reactive substance (TBARS) and efficacy of antioxidant defense system**.** To prepare 10% (*w*/*v*) testis homogenate, testis were separated from their outer tunica albuginea and parenchyma was suspended into ice cold PBS and homogenized in a glass homogenizer, centrifuged for 30 min at 10,000 × g at 4 °C. The supernatant was separated and stored at -80 °C or further biochemical assays. Testicular oxidative stress level was measured spectrophotometrically using thiobarbituric acid reactive substances (TBARS), as described by Ohkawa *et al.* (1979). The protein content of the supernatant was determined using the method described by Lowry *et al.* (1951).

To determine testicular superoxide dismutase (SOD), catalase (CAT), Glutathione S-transferase (GST) and reduced glutathione (GSH) activity method described by of Asada, (1974), Aebi *et al.* (1984), Habig *et al.* (1974) and Rahman *et al.*, 2007 was adopted. Obtained data was calculated from a standard curve and expressed as µmol/mg of protein.

Assessment of sperm parameters

The main purpose of sperm quality analysis for any drug is to evaluate the associated side effect of exposed drug to experimental animal. It is very powerful tool in reproductive toxicity research area. Cauda epididymis was used to assess sperm parameters such as motility, viability, morphology and concentration whereas for daily sperm production in testis is done with testis tissue. To evaluate percentage

of sperm motility in cauda, WHO laboratory manual, (1992) has been adopted. A viability study (percentage of live spermatozoa) was done using eosin/nigrosin stain (Singh *et al.*, 2000). Robb *et al.* (1978) has adopted for evaluating the sperm concentration in epididymis. To evaluate daily testicular sperm production, the procedure described by WHO laboratory manual, (1992) has been adopted. The method described by Wyrobek and Bruce, (1975) has adopted to evaluate the sperm abnormality. Same cauda sperm suspension was used for sperm DNA analysis (Chohan *et al.*, 2004).

Estimation of serum LH, FSH, testosterone and estradiol assay

The estimation of serum LH and FSH carried out by the method described by respected DSI convention (RH 152, LOT 122042 and RH 151, LOT 120038). The estimation of serum testosterone hormone was carried out by the method described by human ELISA kit (cat# AA E-1300, Labor Diagnostika Nord, GmbH, Am Eichenhain, Nodhorn, Germany) whereas for serum estradiol, method described by DiaMetra kit has followed (Ref., DKO003, LOT 4511A).

Histopathological and immunohistochemical study

For histopathological and immunohistochemical perspective studies, Bouin's fixed testis were processed through the sequential changes of alcohol (70%, 90%, 90%, 100% and 100%) and cleared with xylene followed with conventional paraffin embedding, sectioned at 5µm. Futhermore, sections were dehydrated in alcohol and xylene, and stained with Hematoxylin and eosin as described by Bancroft and Gamble, (2002). For a detailed view of diosgenin effect on testicular function, same

histological stained (Hematoxylin and eosin) slide were used to encounter the potential of sperm production in each group by means of MSTD (mean seminiferous tubule diameter), JTBS (Johnson's testicular biopsy score) and quantification of germ cells and Sertoli cells in each sections For immunohistochemical analysis, same histological slide were blocked with endogenous peroxidase (3% H_2O_2) and incubated with respected antibody (Santa Cruz Biotechnology). Encountered antibodies with respective antibody were exposed with DAB (3, 3?-diaminobenzidine) and number of positive cells were counted and photographed by using a light microscope (Leica DM 2500) with a digital camera (model-DFC 450C) (Leica Microsystems, Wetzlar, Germany) at 40 × magnification.

Statistical analyses

Statistical analyses for all obtained parameters were subjected to one way ANOVA with Turkey post hoc multiple comparison test (parametric data) using SPSS for Windows (SPSS, Inc. Chicago, IL, USA, ver. 20.0). Alphabet between columns signify that mean ± SEM with different letters considered to be statistically significant at $p < 0.05$ while alphabet with the same letter do not differ significantly at $p < 0.05$.

Summary

- Acute toxicity study of diosgenin revealed that a single dose of diosgenin at 100, 200, 400 and 600 mg/kg/b.w does not significantly alter the hepatic and renal functions.
- Diosgenin exposure elevated the hepatic and renal function and increases the disorder of lipid metabolism as there was increase in cholesterol and triglycerides level whereas serum HDL and LDH were significantly decreased.
- Diosgenin has been shown as anti-spermatogenic drugs/agents as there was decline in percentage of motility as well viability in mice.
- Daily sperm production and epididymal sperm concentration were also severely affected with diosgenin exposure.
- Diosgenin significantly increases the morphological abnormalities and sperm DNA damage possibly via increased oxidative stress or hormonal imbalance.
- An imbalance in serum LH, FSH, testosterone and estradiol significantly affected the proliferation and maturation of germ cell as well spermatozoa.
- Diosgenin induces oxidative stress mediated toxicity that influence abnormal physiological changes due to elevated MDA content has been observed in this study.
- Due to excess generation of ROS in testicular tissue, there is imbalance in ROS and antioxidant defense system leading into disrupted testicular function in diosgenin treated groups.

- Diosgenin significantly depleted the enzymatic and non enzymatic antioxidants status (SOD, GSH, GST and Catalase).
- Diosgenin increases the interstitial spaces between seminiferous tubule leading into uneven distribution of Leydig cells that adversely affect testosterone biosynthesis due to poor connectivity with blood vessels.
- Diosgenin exposure enhances the shrinkage of seminiferous tubule leading into a decreased in Johnson's score (assessment of spermatogenesis).
- Loss of weight as well pathological changes in ultra structure of cauda epididymis and seminal vesicle due to oxidative stress or reduced testosterone could be the one of reasons behind the abnormal sperms.
- Diosgenin not only disrupted testicular steroidogenesis, it also altered the adrenal cortiosteroidogenesis as there was decline in volume density of cortical area.
- Diosgenin administration has shown to suppress the cholesterol transport to inner mitochondria membrane as there was reduced expression of StAR protein in testicular tissue.
- Diosgenin, an exogenous steroid/drug interferes in endocrine function as well as steroidogenic enzymes that leading into suppression of 3β-HSD and LHR expression.
- Diosgenin has shown to upregulate the aromatase activity suggesting its interference in aromatase receptor.
- Diosgenin exposure induces the testicular atrophy leading into degeneration of testicular tissue and poor blood supply leading excessive cell death in tubule.

- Interrupted spermatogenesis due to diosgenin interference significantly increases the apoptotic rate via abnormal protein expressions of Bcl-2 and caspase-3 genes.
- Use of diosgenin for a longer duration can make sever fertility issue as herein we observed a decline in percentage of fertility potential as well decrease in litter numbers.

Limitation of the thesis and further approach to work

Based on the above observations and limitations of our thesis, we investigated diosgenin effect on hormonal, physiological, metabolic and some testicular protein expressions. Although, there has been a lot of experiments on animal model for therapeutic purposes, such as anti-diabetic, anti-inflammatory, anti-proliferative, anti-depressive and anti-aging etc, but its toxicity part is ignored. Previous and recent reports suggested that steroid may show harmful effect on reproductive function. Based on it, we are further interested to find out mechanism behind diosgenin related testicular toxicity at molecular as well as gene level. We also want to clarify whether change in exposure system (*in vitro* and *in vivo*) of diosgenin changes its efficacy or not. Maximum steroid treatment is done subcutaneously to limit its metabolism in digestive tract. Hence, change in route of exposure, diosgenin would be in direct contact of blood which may show some other changes in body. Furthermore, most interesting question which arises in our mind is that maternal exposure of diosgenin in gestational period would cause any reproductive alteration or related issue in offspring later in life or not.

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