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Research Article

ANTIOXIDANT AND PROTECTIVE EFFECT OF AQUEOUS EXTRACT OF *ICHNOCARPUS FRUTESCENS* AND *CYPERUS ROTUNDUS* AGAINST CISPLATIN INDUCED TESTICULAR TOXICITY IN RODENTS

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ABSTRACT

In the present investigation, the aqueous root extract of *Ichnocarpus frutescens* (*AREIF*) andaqueous rhizome extract of *Cyperus rotundus (ARECR)* was studied for anti testicular toxicity in rodents. A number of agents have been used for the induction of testicular toxicity. Rats have been used as a suitable species for study of anti-testicular toxicity study because of its reproductive system resembling closely that of the human. In this study, Cisplatin (10mg/kg. p.o.) induced testicular toxicity model was used. Experimental induction of testicular toxicity resulted in dysfunction of testicular cells in testis of experimental animals. The major reason for testicular cell dysfunction is due to formation of platinum radicals which damages testicular cells. Results of cisplatin administration indicate the histological changes and the alteration in the amount of free radicals and caused the decrease in sperm count, sperm motility, reduced testosterone and alteration of oxidant levels. After treatment with *AREIF* and *ARECR* at the dose levels of 100, 200 and 400 mg/kg p.o. significantly restored the physiological and also histological changes. These results indicate the beneficial effect in preventing dysfunction of testicular cells.

Keywords: Cisplatin; *Cyperus rotundus; Ichnocarpus frutescens*; Sperm count; Testicular toxicity; Testosterone.

INTRODUCTION

The primary functions of the male reproductive system are the production of sperm, the transportation of sperm from the testes out of the male body, placement of sperm into the female's vagina and the production of glandular secretions and hormones. Toxicants which affect the male reproductive system can act either directly or indirectly and each class of toxicant may require biotransformation prior to its action (Peter k., 1989). Some agents act directly because of their chemical reactivity. Those which act indirectly do so because of their similarity to some endogenous compound or because they cause an alteration in a physiological control mechanism important to reproduction. An indirect reproductive toxicant is defined as an agent which acts at a non-germ cell site to alter the hormonal control of the testis and thus alter reproduction. In contrast an agent which affects the testis without endocrine mediation is classified as a direct reproductive toxicant. Highly reactive intermediates produced in the liver are not likely to reach the testes in significant concentration because of their short biological half-lives (Nelson SD *et al*., 1977). Conversely less reactive metabolic products of hepatic metabolism may attain significant concentrations in the testes and presumably be further metabolized to more toxic forms.

The testes contain measurable amounts of cytochrome P_{450} mixed-function oxidases, epoxide hydrases aryl hydrocarbon hydrolases and the various transferases necessary for the biotransformation of many exogenous compounds (Dixon RL *et al*., 1980; Heinrichs WL *et al*., 1980) although these enzymes and cofactors are often present at only a fraction of their concentration in the liver. In fact the interaction between gonadal activation and hepatic detoxification of xenobiotic likely plays a significant role in the modulation of testicular toxicity. Some reproductive tract toxicants specifically interfere with normal endocrine function but most have multiple mechanisms of actions and targets. Root of *Ichnocarpus frutescens* and rhizome of *Cyperus rotundus* were already reported for potent antioxidant property. Hence in order to identify and correlate the prior claims as an active drug with negligible adverse events this study was conducted.

MATERIALS AND METHODS

Animals

Healthy adult male albino rats of wistar strain weighing 150-120gms were selected for the study. The animals were acclimatized to standard laboratory condition with temperature 25±2ºC and fed with standard animal pellet feed (Sai Meera Foods Pvt. Limited) and water *ad libitum.* The protocol was approved by animal ethics committee as per CPCSEA guidelines. (Ref.No:XII/VELS/PCOL/24/2000/CPCSEA/IAEC/11.03.11).

Plant material

The fresh roots of *Ichnocarpus frutescens* were collected from local areas of Tirupathi, Andhra Pradesh and the fresh rhizomes of *Cyperus rotundus* were collected from local areas of Chennai, Tamil Nadu, India. The plant parts were authenticated by Dr. P. Jayaraman, PARC, Botanical Survey of India, Chennai, Tamil Nadu. They were dried under shade and ground to get a coarse powder.

Preparation of extract and Stock solution

The aqueous extracts (*AREIF* and *ARECR*) of root and rhizome were prepared by using pure water, by maceration method for 72hrs at room temperature. The extracts were concentrated by simple evaporation at room temperature. A suspension of AREIF and ARECR in 2% (w/v) carboxy methyl cellulose was prepared for oral administration. The stock solution concentration was 200mg/ml and used throughout the study.

Phytochemical study

The various phytochemical testing for Alkaloids (Mayer's test, Dragendroff's test), Carbohydrates and Glycosides (Molisch's test, Brontrager's test), Cardiac Glycosides (Legal's test, Keller-killani test), Sugars (Fehling's test, Benedict's test), Steroids (Libermann's test, Salkowski test), Proteins (Millon's test, Biuret test, Ninhydrin test, Xanthoprotein test), Terpenoids (Muller's test), Flavonoids (Shinoda test),Tannins, Anthocyanin, Quinones was performed. (Basset *et al*., 1985; Hebert *et al.,* 1984; Harbourne 1984; Kokate *et al.,* 1990)

Acute toxicity study

The acute oral toxicity study was carried out as per the OECD guidelines-423. Animals were observed individually after administration of *AREIF* and *ARECR,* during the first 30 minutes, and periodically 24 hours with special attention given during the first 4 hours and daily thereafter for a total of 14 days for toxic symptoms and mortality. All observations are systematically recorded with individual records being maintained for each animal. One-fifth, one-

tenth and one-twentieth dose of the maximum dose used in the acute toxicity study was considered as therapeutic dose for further pharmacological study.

Anti-testicular toxicity study

In the present study, male rats were selected to induce testicular toxicity because the reproductive system of male rats resembles that of humans. The healthy adult male rats were divided into eight groups consisting of six animals and all the drugs were treated orally. Group I was served as untreated normal control, Group II was treated with Cisplatin (10mg/kg p.o.) served as control and Group III-V treated *AREIF* with 100, 200 and 400mg/kg respectively, Similarly, Group VI- VIII treated with *ARECR* 100, 200 and 400mg/kg respectively. After the respective treatment schedule, the blood was collected from all the group animals through retro orbital vein and serum was separated by centrifugation and kept at -20ºC for analysis and were autopsied. The animals were weighed and autopsied under pentobarbital anesthesia (50mg/kg given intraperitoneally) 24 hours after the last dosing of the respective treatment. The reproductive and non-reproductive tissues were separated and used for biochemical analysis.

Epididymal sperm concentration and motility

Spermatozoa in the epididymis were counted as described by Prasad *et al.*,1972. Briefly, the epididymis was dissected and minced with anatomical scissors in a petri dish in 1 ml of Ham-F-10 solution. Both sperm concentration and motility were determined at room temperature (28°C). Progressive motility was evaluated using a light microscope and classified as either motile or non-motile for the purposes of this study. The microscopic field was scanned systematically and each spermatozoa encountered was assessed. The procedure was repeated twice and the average reading was taken. Total sperm number (count) was determined using a haemocytometer. A dilution ratio of 1:20 from each well-mixed sample was prepared by diluting 50µl of epididymal spermatozoa suspended in physiological saline with 950µl diluent. Both chambers of the haemocytometer were scored and the average count calculated, provided that the difference between the two counts did not exceed 1/20 of their sum that is, less than 10% difference. When the two counts were not within 10%, they were discarded, the sample dilution re-mixed and another haemocytometer prepared and counted.

Biochemical measurements

The testicular tissue was homogenized in a Teflon-glass homogenizer with a buffer containing 1.5% potassium chloride to obtain 1:10 (w/v) whole homogenate. The testicular tissue lipid peroxidation level was measured according to modified thiobarbituric acid (TBA) method (Buege *et al.*,1978). MDA reacts with thiobarbituric acid to give a red compound absorbing at 532 nm. The stock reagent contains 2ml 15%w/v trichloro acetic acid, 0.375% w/v thiobarbituric acid and 0.25mol/L hydrochloric acid. 0.5g of testicular tissue sample was homogenized in 5ml of 0.15M KCl and the homogenate centrifuged at 1000g for 10 min in a laboratory centrifuge and the supernatant collected. An aliquot of 2ml of the stock reagent was added to 1ml of testicular homogenate supernatant and mixed thoroughly and placed in an Equitron water bath (80 - 90°C) for 15min. It was then cooled and the flocculent precipitate removed by centrifugation at 1000g for 10min and the absorbance of the supernatant determined with a spectronic spectrophotometer at 532nm against blank containing all the reagents. Concentration of malondialdehyde was calculated using the molar absorptivity coefficient of malondialdehyde which is $1.56\times105M^{-1}cm^{-1}$. The amount of produced malondialdehyde (MDA) was used as an index of lipid peroxidation. The reduced glutathione (GSH) level in testicular tissue was estimated as described in (Rukkumani *et al.,* 2004). Briefly, to the homogenate, 10% trichloroacetic acid, TCA was added and centrifuged. 1.0 ml of supernatant was mixed with 0.5 ml of Ellmans reagent, 19.8 mg of 5, 5-dithiobisnitro benzoic acid DTNB (Sigma Aldrich Inc., St. Louis, Mo, USA Batch D8130-5G) in 100 ml of 0.1% Sodium nitrate and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412nm on the spectrophotometer. The level of GSH was expressed as µmol/ml. Catalase was assayed colorimetrically at 620nm and expressed as µmol of H_2O_2 consumed/min as described in (Rukkumani *et al.,* 2004). Briefly, the reaction mixture of 1.5 ml contained 1.0 ml of 0.01M pH 7.0 phosphate buffer, 0.1ml of tissue homogenate and 0.4 ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent, 5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratios.

Hormonal assays

Blood samples were collected into a heparinized bottle and centrifuged at 3000 rpm for 15 min using a bench centrifuge and the plasma stored at 4°C for subsequent assay of Testosterone (TT), Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) according to the method described by Jaswant Singh *et al.*, 1988. The samples were collected in the morning to reduce the influence of diurnal variation in hormones. For all estimations, a minimum of 8 to 10 replicates was done for each parameter.

Statistical analysis

All the values are expressed as mean ± S.E.M. The data were statistically analyzed by one-way ANOVA followed by Dunnet-t test. $P < 0.01$ was considered significant.

RESULTS AND DISCUSSION

Preliminary phytochemical study

Preliminary phytochemical studies revealed the presence of alkaloids, flavonoids, glycosides, triterpenoids and steroids in AREIF and ARECR showed the presence of alkaloids, glycosides, steroids, proteins, flavonoids, triterpenoids and carbohydrates.

Acute toxicity

The purified and completely dried yields of AREIF and ARECR was subjected for the acute oral toxicity study to determine the therapeutic dose using albino mice in controlled environment. No deviation from normal behavioural pattern was observed. But only few animals showed mild behavioural changes like dyspnoea and mild writhings in higher dose. Observation was done continuously for 14days and no mortality was produced in any of the drug treated group. No toxicity was exhibited by both the extracts up to the dose level of 2000mg/kg., p.o.

Antitesticular toxicity study

Recently extracts of plants have provoked interest as sources of natural products. They have been screened for their potential uses as alternative medicines for the treatment of many diseases. (Suman Acharyya *et al*, 2010). Interestingly, although a number of active principles like alkaloids, glycosides, steroids, flavonoids, tannins and terpenoids have been identified from AREIF and ARECR. No attempt appears to have been made so far to determine the antitesticular toxicity effects of these extracts in animal models. The male reproductive system consists of the testis as the main reproductive organ and other accessory structures, with a primary responsibility of sperm production. Agents (especially oxidative agents) that alter testicular function will affect the quality and quantity of spermatozoa, which depends on several reproductive factors. In the present study, administration of 10mg/kg dose of Cisplatin in normal saline solution to male Wistar rats resulted in testicular toxicity was confirmed with the deviations in the levels of reproductive hormones and semen parameters. The reproductive hormones studied were testosterone, FSH and LH, while the semen parameters were sperm count, sperm motility, sperm morphology, sperm debris and primordial sperm count.

Antioxidant parameters

In a situation of oxidative stress, reactive oxygen species, such as superoxide (O2.), hydroxyl (.OH) and peroxyl (.OOH, ROO.) radicals are generated. The reactive oxygen species play an important role related to the degenerative or pathological processes. (Sachin S Sakat *et al*, 2010). Free radicals in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are implicated in numerous pathological conditions. ROS such as hydroxyl radicals (-OH), super oxides (O_2, -OOH), and hydrogen peroxide (H_2O_2) are by-product of normal metabolism. Living system is therefore protected from ROS and RNS by antioxidant enzymes. (Nilesh Babre *et al*., 2010).

Table 1: Effects of AREIF and ARECRfollowing Cisplatin administration on testicular biochemical parameters

Groups	MDA (µmol/mg Protein)	CAT (µmol/min)	GSH (µmol/ml)
Normal	0.876±0. 083	20.081±0.387	0.037±0.0037
Control	9.912±0.7206	0.692±0.022	0.027±0.0023
Test I (AREIF100 mg/kg)	2.889±0.173 [a,b]	18.444±0.336[b,c]	0.034±0.0026[b]
Test II (AREIF200 mg/kg)	1.386±0.131 [b,c]	19.612±0.501[b]	0.041±0.0013[b]
Test III (AREIF400mg/kg)	0.668±0.035[b]	21.601±0.278[b,c]	0.048±0.0026
Test I (ARECR100 mg/kg)	4.464±0.118 [a,b]	18.728±0.351[b,c]	0.030±0.0019
Test II (ARECR200 mg/kg)	2.011±0.194 [a,b]	19.651±0.253[b]	0.0411±0.0018[b]
Test III (ARECR400mg/kg)	0.568±0.033[b]	20.437±0.262[b]	0.051±0.0016 [a,b]

Values are expressed as mean ±S.E.M (n=6), One-way ANOVA followed by Dunnett-t test.

[a]P < 0.01:[c]P < 0.05; Groups (3-8) Vs Group 1; [b]P < 0.01; Groups (3-8) Vs Group 2; MDA:malondialdehyde; CAT: catalase activity; GSH: reduced glutathione.

The treatment of AREIF and ARECR significantly reduces (p<0.01) the elevated levels of MDA towards normal in dose dependent manner. Similarly, CAT level was significantly restored (p<0.01) in all the test drug treated groups when compared with the normal and control group. In contrast, the GSH levels were remarkably (p<0.01) altered to near normal but it is highly significant in lower dose level compared to control.

Semen Parameters

The oral administration of the AREIF and ARECR resulted in a significant increase in the number of spermatids and spermatozoa present in the tissue. Sperm count was reversed to normal at higher dose (p<0.01) treatment of both AREIF and ARECR treatment compared to control.

Table 2: Effects of AREIF and ARECR on Sperm count and motility in rats

Groups	Parameters	
	Sperm Count ($\times 10^6$ / ml)	Sperm Motility (%)
Normal	133.5±3.528	82.667±3.593
Control	67.167±2.971	39.833±5.879
Test I (AREIF100 mg/kg)	106.17±2414 [a,b]	74±2.582[b]
Test II (AREIF200 mg/kg)	112.83±4.191 [a,b]	81.667±1.892[b]
Test III (AREIF400 mg/kg)	130.17±2.833[b]	94.167±1.956[b,c]
Test I (ARECR100 mg/kg)	107.17±3.400 [a,b]	72.833±1.579[b]
Test II (*ARECR* 200 mg/kg)	122±2.921[b]	82.167±1.701[b]
Test III (ARECR400 mg/kg)	137±2.966[b]	94±3.907[b,c]

Values are expressed as mean ±S.E.M (n=6), One-way ANOVA followed by Dunnett-t test.

[a]P < 0.01:[c]P < 0.05; Groups (3-8) Vs Group-1; [b]P < 0.01; Groups (3-8) Vs Group-2.

The increase in sperm density and motility in cauda epididymis is of importance with regard to fertilization. Therefore, the AREIF and ARECR caused an androgen stimulatory effect on the target organs, beneficial alterations in the motility, morphology and metabolism of the spermatozoa in male rats. Sperm motility was enhanced at all the dose levels of AREIF and ARECR treatment.

The increase in the cauda epididymal sperm motility might be due to an alteration in the microenvironment in the cauda epididymis of the treated rats may be as a result of the androgen-stimulatory effect. The increase in the cauda epididymis sperm count in the treated animals substantiates the spermatogenic nature of the extracts. The extract had a direct effect on the testes resulting in an increase in the number of spermatozoa and the increased level of testosterone production.

Reproductive Hormones

Testosterone, the major androgen, is necessary for fetal male sexual differentiation, pubertal development, and the maintenance of adult secondary sex characteristics and spermatogenesis. Testosterone also regulates gene expression in most extra genital tissues, including muscle and bone, and the immune system. The testes are the source of more than 95% of the circulating testosterone in men although the adrenal cortex produces large amounts of the testosterone precursor steroids, dehydroepiandrosterone and androstenedione. (Stephen J, 1988).

Table 3: Effects of AREIF andARECR treatment on hormonal parameters in rats.

Groups	TT(ng/ml)	LH (mIU/ml)	FSH (mIU/ml)
Normal	0.851±0.018	83.667±1.926	86.167±2.75 [a,b]
Control	0.456±0.0212	141.5±2.604	187.5±2.930 [a,b]
Test I (AREIF100 mg/kg)	0.693±0.0248 [a,b]	105.83±1.833[a,b]	148.17±2.668 [a,b]
Test II (AREIF200 mg/kg)	0.786±0.018[b]	101.17±1.424 [a,b]	130.67±2.704 [a,b]
Test III (AREIF400 mg/kg)	0.855±0.015 [b]	88.66±1.892[b]	104.83±2.007 [a,b]
Test I (ARECR100 mg/kg)	0.661±0.016 [a,b]	108±2.16 [a,b]	138.17±2.561 [a,b]
Test II (ARECR200 mg/kg)	0.796±0.014[b]	100.67±2.155 [a,b]	119.83±1.973 [a,b]
Test III (ARECR400 mg/kg)	0.875±0.031[b]	83.833±2.994[b]	101.67±1.626 [a,b]

Values are as mean ±S.E.M (n=6), [a]P < 0.01:[c]P < 0.05; Groups (3-8) Vs Group-1; [b]P<0.01; Group (3-8) Vs Group-2. TT:Testosterone; FSH: Follicle stimulating hormone; LH: Leutinizing hormone.

The results in this study showed significantnormalisation in testosterone level was exhibited by AREIF 400mg/kg alone. The extracts did not show an antigonadotrophic nature, demonstrated by the increased level of FSH levels in the treated rats. The increased

level of FSH reveals a possible role of extracts in influencing the release of gonadotrophic hormones from the pituitary. The rise of FSH by itself is of critical importance in the initiation and expansion of spermatogenesis in mammals, as is generally agreed. (Sharpe M. 1989). Leutinizing hormone levels were reversed to almost normal level only in higher dose AREIF and ARECR treated groups compared to control.

The histological report of the testes revealed the protective effect of the test drugs at the different dose levels.

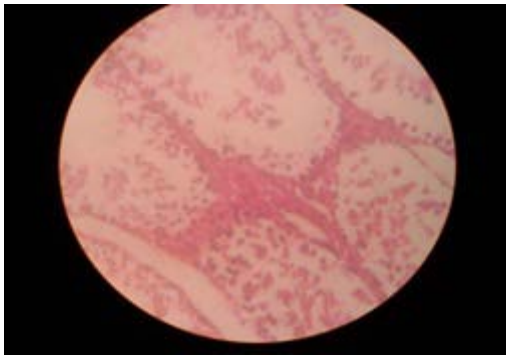

Fig. 1: Normal (Shows spermatogenesis)

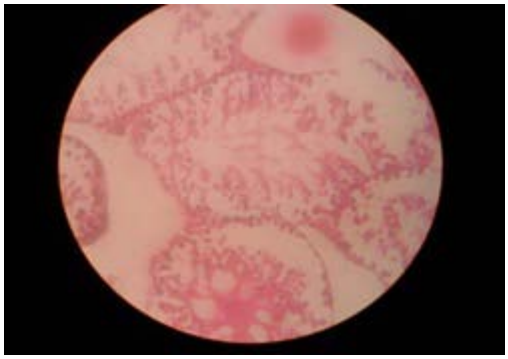

Fig. 2: Cisplatin treated (Spermatogenesis arrested)

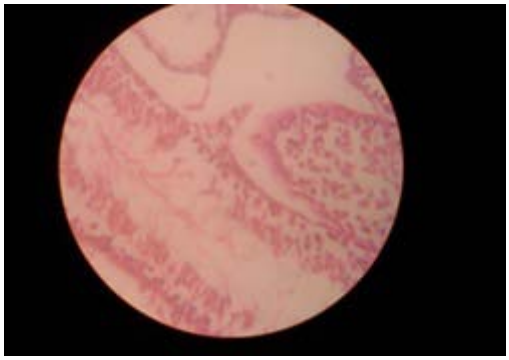

Fig. 3: AREIF100 mg/kg treated (Spermatogenesis partially arrested)

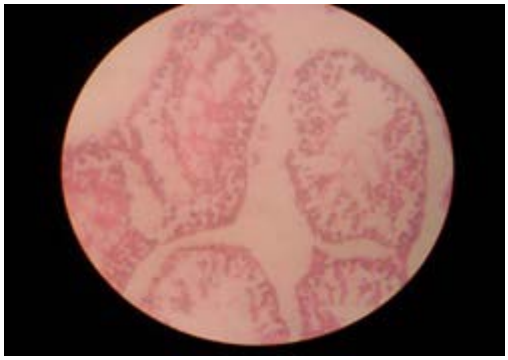

Fig. 4: AREIF200 mg/kg treated (Near normal spermatogenesis)

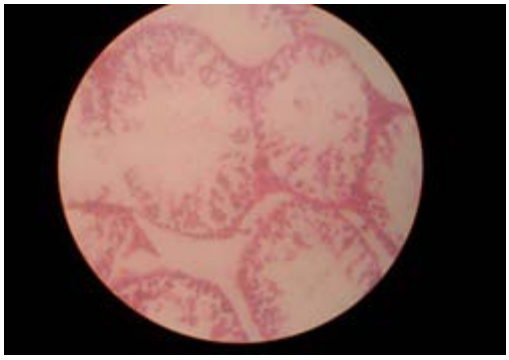

Fig. 5: AREIF 400 mg/kg treated (Normal spermatogenesis)

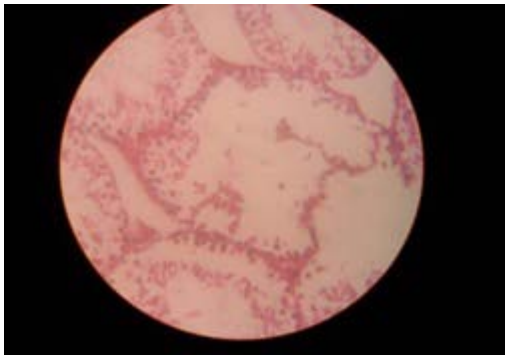

Fig. 6: ARECR100 mg/kg treated (Spermatogenesis partially arrested)

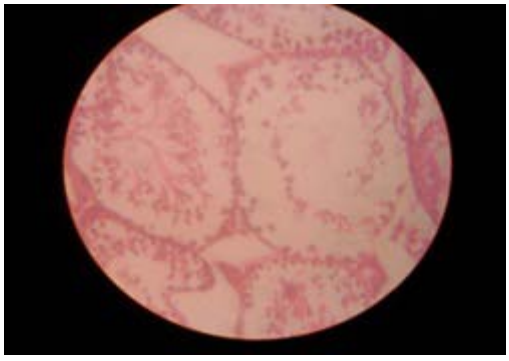

Fig. 7: ARECR200 mg/kg treated (Normal spermatogenesis)

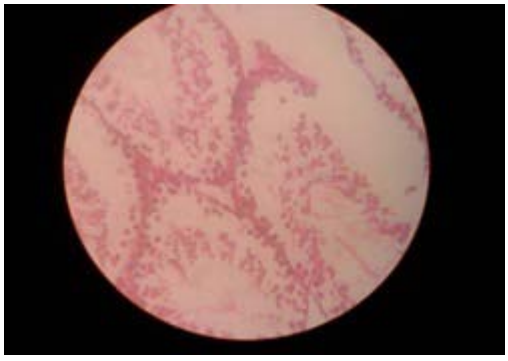

Fig. 8: ARECR400 mg/kg treated (Normal spermatogenesis)

CONCLUSION

The phytochemical investigation of AREIF revealed the presence of alkaloids, carbohydrates, glycosides, saponins, tannins, phenolic compounds, flavonoids, triterpenoids and phytosterols whereas the phytochemical investigation of ARECR revealed the presence of alkaloids, carbohydrates, glycosides, saponins, tannins, flavonoids, tannins, proteins and triterpenoids. From the acute toxicity study, it was confirmed that the test drug AREIF and ARECR were practically nontoxic on oral administration. The severity of microscopic testicular tissue correlated well with free radical concentration in testis. After treatment with AREIF and ARECR (100, 200 and 400mg/kg respectively) showed increase in sperm count, sperm motility, testosterone and restoration of antioxidants.

These results indicate the beneficial effect in preventing dysfunction of testicular cells. The aqueous extracts of *Ichnocarpus frutescens* and *Cyperus rotundus* showed significant decrease in FSH, LH concentrations in testes. In the current investigation, histopathological evaluation showed the maximum prevention of testicular cell damage at the dose of 400mg/kg compared to 100 and 200mg/kg which may be due to the active compounds that are present in aqueous extract showing activity at higher dose. The extracts treatment restored normal functioning of testicular cells. Extract-treated group remarkably increased the sperm count which is indicative of improvement in testes function. The mechanism of antitesticular toxicity activity of aqueous extracts may involve the inhibition of free radical production along with enhancement of the body defense system. AREIF and ARECR showed promising dose–dependent antioxidant activity in all the parameters tested. Thus it can be concluded that drug-treated group showing cytoprotection due to its effect on prevention of free radical production in testicular cells so the mechanical disruption of epithelium is less or protection against free radicals rearrangements**.**

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