JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Reproductive toxicological effects of cafeteria diet, alcohol and their combination on sperm quality parameters, steroidogenic enzyme activities, testicular antioxidantcapacity in male wistar rats

[1]Rupendra G, [2]Sreenivasula Reddy P*

[1]Research Scholar,[2]Professor
[1]Department of Biotechnology, [2]Department of Zoology
[1,2] Sri Venkateswara University, Tirupati, India

Abstract : The present study aimed to investigate individual and combined of cafeteria diet combined alcohol were evaluated for reproductive toxicities in male wistar rats. A total of twenty-four rats were divided into four groups. 1st administered normal diet, 2nd administered cafeteria (CAF) diet, 3rd administered alcohol alone (2.0 g/kg/bw/day) and 4th administered cafeteria diet combined alcohol for 60 days. Compared with control diet group the rats of CAF diet treated group showed improved body weight gain. Further, increased bodyweight gain in Co-administration of CAF diet combined alcohol intake rats compared to control rats. Conversely, alcohol treated rats did not show any significance in body weight compared to control. The relative weights of testes, cauda epididymis, seminal vesicles, vas deferens and prostate glands were significantly decreased in alcohol alone or combined cafeteria diet exposure rats compared to control rats ($P < 0.05$). The CAF diet reduced daily sperm production, sperm motility, viability, HOS-tail swelled sperm, while alcohol treated rats also displayed decreased these parameters when compared to control rats. Co-administration CAF diet combined alcohol intake resulted in poor sperm quality parameter. Regarding activities levels of serum testosterone and steroidogenesis marker enzymes of 3 β-HSD, 17 β-HSD were significantly reduced by CAF diet or combined alcohol treated rats. Our results also displayed that there was significant decrease of antioxidant enzyme (SOD, CAT, GSH-GGPx, and GR) activities and remarkable elevated in MDA content in testes in rats exposed to CAF diet or alcohol alone. Further, adversely depressed activities of antioxidant enzymes and increase lipid peroxidase content in co-administrated CAF diet plus alcohol intake can increase oxidative stress in testes. In summary, these results indicated that CAF diet combined alcohol intake rats can suppress spermatogenesis and testicular steroidogenic marker enzyme of 3β-HSD and 17β-HSD and this effect may be attributed to its higher reproductive toxicity than individual treatments. Further, also affect the testosterone levels in serum and testicular steroidogenesis marker enzymes of 3β-HSD and 17β-HSD were decreased in rats can increase higher reproductive toxicity than individual treatments. Thus, CAF diet and alcohol synergetic effect led to adverse effect on male reproductive system.

IndexTerms **- Alcohol; Obesity, Toxicity; Cafeteria diet**

I. INTRODUCTION

Lipid metabolic disorders mainly due to poor life style eating habits are on the rise in both developed and developing countries, with a negative impact of the “Western diet” on male infertility through decrease sperm quality and motility [1]. A number of studies have also suggested that decreased testosterone level in serum and 3 β-HSD and 17 β-HSD steroidogenic marker enzyme activities in testes. A high body mass index and fat accumulation promote oxidative stress. Nevertheless, the mechanisms how high fat diet affects male reproduction remain unclear, so more studies are required. Consumption of alcoholic beverages has been an integral part in many cultures since beginning of ancient history and also considered as lifestyle diseases. Alcohol abuse has been linked to several disease such as neuropsychiatric problems, cardiovascular, gastrointestinal, unintentional injuries, cancer, diabetes mellitus, fetal alcohol syndrome, infectious diseases, and reproductive disorders and contribute to alcoholism related morbidity and mortalit. Previous studies have demonstrated that exposure of rodents to alcohol results in significant decreased testicular weight, accessory sex organ weights [2,3,4]. Moreover, alcohol reportedly elicits toxicity via generation of reactive oxygen species (ROS) which leads to morphological deterioration in testis, reduced steroidogenic enzyme activities [5], testosterone deficiency [6]. Furthermore, the alterations of protein expression in epididymis can lead to decrease sperm quality and sperm maturation [7]. However, the individual evidences addressing the adverse reproductive outcomes resulting from exposures to cafeteria diet and alcohol have been demonstrated, the studies pertaining to combined effect of both in relation to male reproductive health are sparse. It is reasonable to suspect, that at times, humans are simultaneously exposed to concurrent alcohol combined cafeteria diet, this study focuses on the effect of alcohol and cafeteria diet separately and combined on reproduction in male wistar rats.

II. MATERIAL AND METHODS

2.1 Procurement and maintenance of animals

Male wistar rats with a body weight of 190±10 g (12week old) was purchased from an authorized vendor from Sri Raghavendra Enterprises, Bengaluru, India. All rats were housed (4 per cage) in clean polypropylene cages (18" x 10'x 8") containing paddy husk as bedding material and were provided with tap water ad libitum and standard rodent chow purchased from Sai Durga Agencies, Bengaluru, India. Rats were acclimatized for one week before being used for experimentation and maintained at temperature 22-25°C; 12:12 hr light: dark cycle. The experiments were carried out in accordance with guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, Ministry of Social Justice and Empowerment, Government of India [8].Authors declare that the experiments were consistent with the guidelines and principles of the Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India and approved by the Institutional Animal Ethical Committee at S.V. University, Tirupati, India (vide No. IAEC/No. 438/01/a/CPCSEA) with a resolution No: 57/2012/(i)/a/CPCSEA/IAEC/ SVU/PSR- KPR dt.08-07-2012.

2.2 Experiment Design

The animals were randomly divided into four experimental groups: the Control diet (n = 6; standard rodent chow obtained from Sai Durga Agencies, Bengaluru, India), and the cafeteria diet (n = 6; CAF diet consisted 3.75 g chocolate + 7.5 g biscuits + 7.5 g dried coconut + 6.25 g pellet chow (1.5:3:3:2.5), alcohol (n = 6; rats given as 2.0 g/kg/bw/day), cafeteria diet (CAF) plus alcohol (n = 6; CAF diet consisted 3.75 g chocolate + 7.5 g biscuits + 7.5 g dried coconut + 6.25 g pellet chow (1.5:3:3:2.5) plus 2.0 g alcohol/kg/bw/day) for 60 days. All diet were given ad lithium. The initial body weight on the first day and the final body weight on prior to necropsy of the experiment were determined and used to calculate body weight gain over the 60-day period of treatment. Afterward, the animals were fasted overnight before being euthanized by either anaesthesia and cervical dislocation on the day following the last treatment. Moreover, brain, liver, kidney, testes, epididymis (caput, corpus and cauda), vas deferens, seminal vesicles prostate gland a were quickly removed, cleared of adhering connective tissue, weighed separately by using a Schimadzu electronic balance (Model No: BL-220H; Kyoto, Japan). Tissue somatic indices (TSI) were calculated by using the following formula:

$$TSI = [\text{ weight of the tissue (g) / body weight of the animal (g)}] \times 100$$

Testes were used for determination of daily sperm production (DSP) and biochemical studies whereas epididymides were used for the determination of sperm analysis and biochemical studies. Further, serum was obtained by centrifuged with 3,000 x g for 10 min and stored at -20°C until used for determination of testosterone concentrations.

2.3 Sperm parameters

Daily sperm production was determined in the testes of adult male wistar rats [9]. In brief, the testis was decapsulated and the parenchyma was homogenized in 50 mL of ice-cold 0.9% sodium chloride solution containing 0.01% Triton X 100 using a glass Teflon homogenizer. The homogenate was filtered through a metal sieve to remove connective tissue, and the filtrate was used to count the number of homogenization-resistant spermatids using a Neubauer haemocytometer. The number of sperm produced per gram of testicular tissue per day was calculated. Moreover, sperm were collected from right tail of epididymis in 2.0 ml of M-199 medium with 5 mg/mL bovine serum albumin. Sperm count and evaluation of the motility were done by the previous described method [10]. Sperm count was expressed in millions/mL and motility was expressed as percentage of motile sperm of the total sperm counted. The ratio of live and dead spermatozoa was determined during using 1% trypan blue reagent. Sperm viability was expressed as percentage of unstained sperm of the total sperm counted. The hypoosmotic swelling (HOS) test was performed [11]. The data were expressed as millions/ml for sperm count, and for other sperm parameters, the data were expressed as percentage of total sperm.

2.4 Assay of testicular steroidogenic marker enzymes

The activities of 3β and 17β- hydroxysteroid dehydrogenase (HSD) were measured [12]. Furthermore, the reaction mixture in a volume of 2.0 ml of contained 100 µM of sodium pyrophosphate buffer (9.0), 0.5 µM cofactor NAD for 3- β HSD, 0.5 µM NADPH for 17 β -HSD, 0.08 µM substrate (dehydroepiandrosterone for 3-βHSD, androstenedione for 17β-HSD) and 100 µM of coenzyme. Furthermore, the reaction was carried out in a quartz cuvette of 1.0 cm path length at 23°C and then absorbance at 340 nm was measured at 20 seconds for 3 minutes in an ultraviolet (UV)-Visible double-beam spectrophotometer (UV-1700, Shimadzu, Japan). The enzyme activites was expressed as nM of NAD converted to NADH / mg protein / min for 3β- HSD and as nM of NADPH converted to NADP / mg protein / min for 17β-HSD. Protein content in the enzyme source was estimated [13] using bovine serum albumin as standard.

2.5 Determination of serum testosterone concentrations

The serum testosterone levels were determined by an ELISA method using a DRG testosterone ELISA kit (ELISA EIA-1559, 96 wells kit, DRG instrument, GmbH, Marburg, Germany). The assay was performed according to the user manual. The sensitivity, intra- and inter assay variation coefficients of kit were 0.083 ng/ml, 3.34 - 4.16 % and 4.73 - 9.94 % respectively.

2.6 Estimation of antioxidant indices in testes

A 10% homogenate was prepared with glass-teflon homogenizer in ice-cold Tris buffer (2 mM, pH 7.4, contains 0.25 M sucrose) followed by centrifugation at 800 x g for 20 min at 4°C and clear supernatant was used for enzyme assays. Lipid peroxidation (LGPx) was estimated in terms of malondialdehyde (MDA) content and determined by using the thiobarbituric acid by the method of Ohkawa et al. 1The LGPx levels were expressed as µmol of MDA formed/g tissue. Superoxide dismutase (EC 1.15.1.1) activity was assayed [14]. The SOD activity levels were expressed in nmoles pyrogallol oxidized/mg protein/min. Activity of Catalase (EC 1.11.1.6) was

assayed [15]. The activity of the enzyme was expressed in nmoles of H_2O_2 metabolized/mg protein/min. Glutathione peroxidase (EC 1.11.1.9) activity was measured [16]. The activity was expressed as nmoles NADPH oxidized/mg protein/min. Glutathione reductase (EC 1.6.4.2) was assayed [17]. The activity was expressed in terms of nmoles NADPH oxidized/mg protein/min.Protein content in the enzyme source was determined [13].

2.7 Statistical analysis

The statistical analysis was performed by SPSS 20.0 software (SPSS Inc., Chicago, IL, United State). Data were analyzed by One-way analysis of variance followed by Post hoc Tukey HSD comparison test to identify differences in means among treatments. Data were assumed to be statistically significant at $P < 0.05$.

III Results

3.1 Body weight and reproductive organ weight

After 60 days feeding, the body weight of rats in CAF diet or Co-administrated CAF diet combined alcohol treated group increased significantly compared to control group ($P< 0.05$; Table. 1). In contrast, there was a significantly decreased in body weight gain in alcohol alone treated rats when compared to control rats. The relative weights of testes, seminal vesicles, prostate gland, vas deferens were decreased significantly in alcohol treated rats. CAF diet treatment showed a tendency of alleviate high fat diet induced organ weight gain of testes, epididymis, seminal vesicles, prostate gland, vas deferens was observed when compared to control diet was shown in ($P< 0.05$; Table. 2).

Table 1. Cafeteria diet (CAF) and/or alcohol induced changes on body weights (g) adult male wistar rats

Parameter	CON	CAF	Alcohol	CAF+Alcohol
Initial body weight (g)	200.83±10.3	210.00±6.2*	193.17±2.9*	220.17±6.8*
Final body weight (g)	336.50±5.5	401.83±9.1*	311.75±9.3*	421.67±5.3*
Weight gain (g)	135.67±7.1	191.83±3.0*	118.58±0.1*	201±12.5*

CON = Control diet group; CAF = Cafeteria diet; AL = alcohol; CAF + AL = Cafeteria diet combined alcohol intake. Control rats were provided with standard diet and Cafeteria rats were provided with high fat diet (60% fat). Alcoholic rats were provided alcohol 2.0g/kg/bw/day. CAF rats combined alcohol intake were also supplied to CAF+AL groups. Data are shown as mean ± S.D (Standard Deviation). Significant difference was analyzed using a one-way ANOVA followed by *post hoc* test of Tukey's HSD and indicated by * for $P< 0.05$ compared CONVs CAF, AL, CAF+AL.

Table 2. High fat diet (HFD) and/ or alcohol induced changes on tissue somatic indices (g%) of adult male wistar rats

Parameter	CON	CAF	Alcohol	CAF+Alcohol
Testis	2.03±0.10	2.40±0.56*	1.28±0.13*	3.18±0.16*
Cauda Epididymis	0.18±0.02	0.28±0.06*	0.10±0.05*	0.59±0.26*
Seminal vesicles	0.50±0.28	0.62±0.09*	0.40±0.06*	0.81±0.07*
Prostate gland	0.16±0.05	0.29±0.07*	0.13±0.02*	0.4±0.26*

CON = Control diet group; CAF = cafeteria diet; AL = alcohol; CAF + AL = Cafeteria diet combined alcohol intake. n=6 for male wistar rats per group. Data are shown as mean ± S.D (Standard Deviation). Significant difference was analyzed using a one-way ANOVA followed by post hoc test of Tukey's HSD and indicated by * for P< 0.05 compared CON vs CAF, AL, CAF+AL (treatment groups)

3.2 Sperm quality parameters

The daily sperm production, sperm motility, sperm viability, sperm morphology is presented in Table 3. The alcohol treatment decreased daily sperm production (DSP)in testis, when compared with control group ($P< 0.05$). Likewise, the exposure to alcohol caused a decreased percentage of sperm motility, sperm viability compared to control ($P< 0.05$). Moreover, HOS-tail swelled sperm was statically lower in alcohol treated rats compared to control rats. However, after CAF diet exposure for 60 days obvious impaired spermatogenesis effects were observed. Sperm quality parameters such as daily sperm production, sperm viability, sperm motility was significantly decreased while sperm morphology abnormalities increased in rats subjected to a CAF diet or co-administered with CAF and alcohol ($P< 0.05$; Table 3).

Table 3. Effect of Cafeteria (CAF) diet and/ or alcohol on sperm production in testis and sperm quantity and quality in cauda epididymis of adult male wistar rats

Parameter	CON	CAF	Alcohol	CAF+Alcohol
DSP (millions/g testis)	19.67±2.77	16.30±0.65	14.45±0.74	8.87±0.85
Sperm count(millions/mL)	72.17±0.93	54.63±2.32*	50.03±1.26*	42.80±0.71*
Motile Sperm(%)	75.17±3.09	41.37±0.95*	49.17±4.45*	31.42±1.28*
Viable Sperm(%)	74.73±1.48	47.15±0.55*	51.30±1.37*	32.11±1.20*
HOS-tail coiled sperm(%)	71.50±5.08	35.21±1.04*	40.63±4.35*	28.67±8.03*

CON = Control diet group; CAF = Cafeteria diet; AL = alcohol; Cafeteria + AL = Cafeteria diet combined alcohol intake. DSP = Daily sperm production; HOS-tail coiled sperm = Hypo-osmotic swelling (HOS). Data were expressed as mean ± Standard Deviation (S.D), n= 6 individual/group and * $P< 0.05$. *Compare with respective CON vs treatment groups was done using one way ANOVA followed by post hoc test of tukey HSD multiple comparison test.

3.3 Activities level of testosterone and steroidogenic marker enzymes

Rats fed with alcohol lone presented significantly decreased serum testosterone levels compared with the control group ($P< 0.05$; Table. 4). However, 3β-HSD, and 17 β-HSD are Leydig cell-specific marker decreased significantly in alcohol treated rats ($P< 0.05$). The circulatory levels of testosterone were significantly ($P< 0.05$) decreased in CAF diet or Co-administered with CAF and alcoholrats compared to control rats. The activity levels of 3β- HSD and 17 β-HSDs were also decreased in the testis of these rats (Table 4).

Table 4. Effect of Cafeteria(CAF) diet and/or alcohol on 3β- and 17β-HSD activity levels in the testis and serum levels of testosterone in adult male wistar rats

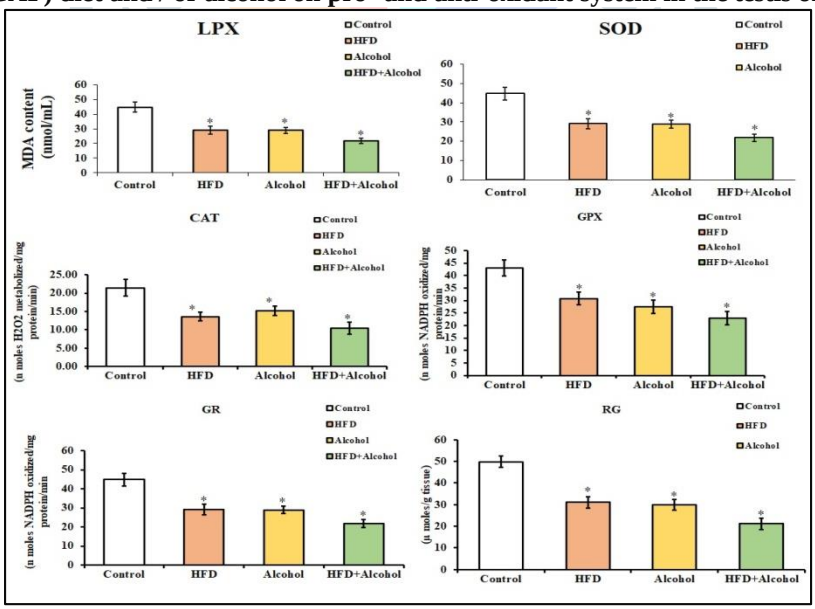
Parameter	CON	CAF	Alcohol	CAF+Alcohol
Testosterone(ng/mL)	2.75±0.45	1.30±0.69*	1.87±1.17*	0.98±0.62*
3β-HSD (nmol of NADPH converted to NADP/mg protein/min)	20.60±1.74	12.22±0.31*	15.93±0.86*	7.00±0.36*
17β-HSD (nmol of NADPH converted to NADP/mg protein/min)	13.28±0.88	8.62±1.19*	9.98±0.78*	6.95±0.27*

CON = Control diet group; CAF = Cafeteria diet; AL = alcohol; Cafeteria + AL = Cafeteria diet combined alcohol intake. Data were expressed as mean ± Standard Deviation (S.D), n= 6 individual/group and * $P< 0.05$. *Compare with respective CON vs treatment groups was done using one way ANOVA followed by post hoc test of tukey HSD multiple comparison test.

3.4 Testicular antioxidant indices

The SOD, CAT, GSH-GPx activities and LGPx and GSH levels of all the treatment groups are shown in Figure 1.

Figure 1. Effect of cafeteria(CAF) diet and / or alcohol on pro- and anti-oxidant system in the testis of adult male wistar rats.



Data were analyzed by One-way ANOVA followed by Tukey HSD *post hoc* test. Bars present Mean ± standard deviation (n = 6); Asterisks (*) indicates significant difference between control and treatment groups at $p < 0.05$. (A) Data clearly shows that there was a significant increase in total antioxidant capacity (T-AOC) in CAF alone or combined treated groupscompared to control (B) There was a significant higher SOD activity in serum of CAF and CAF plus alcohol groups compared to control group (C) The activity level of glutathione peroxidase significantly elevated in CAF or combined groups comparison to control (D) The mean values of GSH content was found to be significantly much higher in treatment group than control (E) Catalase activities was higher in HFD combined alcohol intake exposure rats group comparison to control rats(F) Low serum concentration of malondialdehyde (mean values in n mol) was observed inCAF group, CAF plus alcohol group than control at end of experiment period.

Alcohol administration caused significant ($P< 0.05$) increases in end product of MDA levels, significant decreases in SOD ($P< 0.05$), CAT ($P< 0.05$), GSH-GGPx activities ($P< 0.05$) and GSH levels ($P< 0.05$) of the testes tissue compared with control group. Superoxidase dismutase (SOD) and catalase (CAT), glutathione peroxidase (GSH-GPx) activity levels were decreased in the testis CAF diet treated rats compared to control rats ($P< 0.05$). On the other hand, there was significantly higher MDA and GSH levels in testis as compared control ($P < 0.05$). However, the administration of CAF diet combined alcohol intake (60 % fat and 2.0g/kg/bw/day) adversely increase MDA content and decreases in these antioxidants above mentioned induced by alcohol exposure.

IV Discussion

It is well known that chronic consumption of alcohol may cause alterations in male reproductive function. In the same manner chronic CAF diet causes dysfunction of male reproductive system. However, Co-administration of CAF diet combined alcohol intake in rodent model was remain unknown. Therefore, in the present study, rats were subjected to cafeteria diet or combined alcohol in order to evaluate its effect on the male reproduction. The cafeteria diet used in the present was effective in promoting obesity, as demonstrated by a higher bodyweight. Further, increased in body weight was observed in co-administration of cafeteria diet and alcohol when compared to control rats indicating sign of overt general toxicity. Conversely, alcohol treated rats showed decreased body weight compared to control. The cafeteria diet induced obesity promoted increased bodyweight. In the same manner alcohol treated rats also displayed lower bodyweight [18]. However, cafeteria diet combined alcohol intake was novel findings which suggest that the morphology and functional integrity of testis and accessory sex organs are dependent on availability of androgen. In addition, decrease in the serum level of testosterone may be a reason behind the significant decline observed in weights of testis and accessory sex organs in rats subjected to oxidative stress. These data suggest increased bodyweight and weights of testes, accessory sex organs due to cafeteria diet or combined alcohol intake and also dependent upon testosterone levels which indicating that metabolic change led to sign of toxicity.

The present study also revealed that sperm parameters, the adverse effects associated with cafeteria diet -induced obesity combined alcohol intake are limited documented in the literature. Moreover, in this study, exposure of cafeteria diet (CAF) or alcohol alone resulted in a significant reduction in the testicular daily sperm production (DSP), sperm count, and percentage of motile, viable and HOS tail coiled sperm when compared to that of the control rats. Further, severe adversely effect on sperm parameters such as daily sperm production, sperm count, sperm motility, sperm viability, and HOS tail coiled sperm in co-administration cafeteria diet combined alcohol intake. Earlier studies had reported similar results for cafeteria diet treated rats [19] and alcohol treated rats [20]. Our results additionally showed that cafeteria diet and alcohol combination resulted in poor sperm quality. These data suggest that cafeteria diet combined alcohol intake resulted synergetic effect on male reproductive system by decreased sperm parameter comparatively individual treatment.

In present study data revealed that administration of cafeteria diet or alcohol alone caused a significant reduction in activity levels of 3β-HSD and 17 β-HSD in the testis, and levels of serum testosterone. The present findings are in agreement with several earlier report by Wang and co-workers who reported that decreased testosterone level in serum and activity levels of 3β-HSD and 17 β-HSD in the testis [21]. Co-administration of cafeteria and alcohol treated rats showed similar trends in activity levels of 3β-HSD and 17 β-HSD in the testis and serum testosterone. This finding is novel and significant suggest that the decreased circulatory testosterone level in serum of experimental rats might have led to the degenerative changes in the testis, and androgenic stimulation is required for the normal growth and function of testis.

Antioxidant enzymes such as superoxidase dismutase, catalase, glutathione peroxidase, play important roles in the elimination of ROS, which defend cellular integrity against free radical-induced damages. Oxidative stress has been reported in this study and was in consistent with those observed in other studies. In the present study, exposure to CAF diet or alcohol alone or co-administration of CAF and alcohol induce an increase in the production of MDA and decrease in GSH level and activities of SOD, CAT, and GSH-GPx which may be due to the overproduction of ROS and the deficiency of antioxidant defences, therefore, induced testicular oxidative damage in the testes of rats. Similar results were made individual treatment of cafeteria diet and alcohol effect on testis [22].

References

[1]. Fabrice Saez, Joel R. Drevet. Dietary Cholesterol and Lipid Overload: Impact on Male Fertility, Oxidative Medicine and Cellular longevity, 2019; 11.

[2]. S. D. F. P. Mesquita, M. F. Barbieri, E. V. Fernandes, F. G. Andrade and N. R. Arrebola, Effects of swimming on the testicular histomorphology of alcoholized rats.*Acta Scie Health Sci* 2012;35. 37-42.

[3]. K. R. Muthusami and P. Chinnaswamy. Effect of chronic alcoholism on male fertility hormones and semen quality.*Fertil. Steril*2005; 84. 919 –924.

[4]. WHO. Global status report on alcohol and health.In Management of substance abuse(*Ed.*)World Health Organization. 2014.

[5]. R. Harikrishnan, P. A. Abhilash, S. Syam Das, P. Prathibha, S. Rejitha, F. John. S. Kavitha and M. Indira, Protective effect of ascorbic acid against ethanol-induced reproductive toxicity in male guinea pigs.*Br J Nutr* 2013;110. 689-698.

[6]. P. Hari Priya, B. P. Girish and P. S. Reddy. Restraint stress exacerbates alcohol-induced reproductive toxicity in male rats.*Alcohol* 2014 48. 781-786.

[7]. M. Arco, T. Sartor, R. Cabrera and M. A. Sosa, Prolonged ethanol ingestion decreases alphamannosidase activity and induces its redistribution to the fluid phase in rat cauda epididymis. *Cell Mol. Biol*2003;49. 953-957.

[8]. CPCSEA. CPCSEA guidelines for laboratory animal facility.*Indian J Pharmacol* 2003;35. 257-274.

[9]. W. F. Blazak, K. A. Treinen and P. E. Juniewicz. Application of testicular sperm head counts in the assessment of male reproductive toxicity, in Methods in Toxicology. Male Reproductive Toxicology, ed. R. S. Chapin RE and J. J. Heindel,*Academic Press, San Diego*. CA. 1993. 86-94.

[10]. M. A. Belsey, K. S. Moghissi, R. Eliasson, C. A. Paulsen, A. J. Callegos and M. R. N. Prasad. Laboratory manual for the examination of human semen and semen cervical mucus interaction.*Press Concern*. Singapore. 1980

[11]. R. S. Jeyendran, H. H. Van-Der-Ven and L. J. D. Zaneveld. The hypo-osmotic swelling test: An update.*Arch. Androl* 1992;29. 105-116.

[12]. H. Lowry, N. I. Rosebrough, A. L. Far, R. J. Ranall, Protein measurement with folin phenol reagent. *J. Biol Chem* 195;193. 265-275.

[13]. H. Ohkawa, N. Ohishi and K. Yagi, Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction.*Anal. Biochem* 1979;95. 351-358.

[14]. S. Marklund and G. Marklund. Involvement of superoxide anion radical in autooxidation of pyragallol and a convenient assay for superoxide dismutase.*Eur J Biochem* 1974;47. 469- 474.

[15]. A. Claiborne. Handbook of methods for oxygen radical research, ed. R. Greenwald.*CRC Press*. Florida. 1985;283–284.
[16]. D. E. Paglia and W. N. Valentine. Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase.*J Lab Clin Med* 1967;70. 158-169.
[17]. I. Carlberg and B. Mannervik, Purification and characterization of the flavoenzyme glutathione reductase from rat liver, *J Biol Chem* 1975;250, 5474-5480.
[18]. C. D. B. Fernanadez, F. F. Bellentani, G. S. A. Fernanades, J. E. Perobelli, A. P. A. Favareto, A. F. Nascimento, A. C. Cicogna & W. D. G. Kempinas. Diet-induced obesity in rats leads to a decrease in sperm motility.*Reprod Biol and Endocrinol* 2011;9. 32
[19]. P. Hari Priya, P. Sreenivasula reddy.Effect of restraint stress on lead-induced male reproductive toxicity in rat.*Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*. 2012;317. 455-465.
[20]. G. E. Siervo, H. R. Vieira, F. M. Ogo, C. D. Fernandez, G. D. Goncalves, S. F. Mesquita, J. A. Anselmo-Franci, R. Cecchini, F. A. Guarnier, G. S. Fernandes, Spermatic and testicular damages in rats exposed to ethanol: influence of lipid peroxidation but not testosterone.*Toxicology*. 2015; 330.1-8.
[21]. Er Hui Wang, Zeng Li Yu, Yong Jun Bu, Peng Wei Xu, Jin Yan Xi and Hai Yan Liang, Grape seed proanthocyanidin extract alleviates high-fat diet induced testicular toxicity in rats. *RSC Adv.* 2019; **9**. 11842-11850.
[22]. E. M. Pinto-Fochi, E. Z. Pytlowanciv, V. Reame, A. Rafacho, D. L. Ribeiro, S. R. Taboga, R. M. Goes. A high-fat diet fed during different periods of life impairs steroidogenesis of rat Leydig cells. *Reproduction* 2016;152.795-808.