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Effects of Cycas Circinalis and Ionidium Suffruticosum on Male Wistar Rats that Have Been Made Sterile by Alcohol

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Abstract:
More than eighty million individuals throughout the world struggle with infertility. It's a worldwide phenomenon that cuts across all demographics. Alcoholism is one of today's most pressing social issues because of the wide range of negative health effects associated with heavy alcohol use. Most research on the effects of ethanol on fertility has been done on male humans and laboratory animals. This study aimed to determine whether or not Cycas circinalis and Ionidium suffruticosum might restore fertility in male Wistar rats rendered infertile by alcohol.

1. Introduction

As a serious public health issue, infertility puts stress on any couple's relationship. Because of the emotional and social entanglements, it cannot be treated in the same way as other diseases. It frequently symbolizes the union of the couple's essential masculine and feminine qualities. The failure to conceive after a year of consistent, unprotected sexual activity between couples is considered infertility. Ten percent to fifteen percent of married couples may experience this. About half of these instances involve men, although between fifteen percent and twenty-four percent have a puzzling origin.[1]

Low sperm concentration and sperm motility are blamed for most cases of male infertility. Ejaculate may include immature or defective spermatozoa if spermatogenesis is disrupted. A high percentage of defective spermatozoa might render a man infertile even though his total sperm count is within the usual viable range. One of the strongest factors that might cause infertility in otherwise fertile people is stress. The word "stress" has been used to include a wide range of reactions to unpleasant or potentially noxious stimuli that might heighten the body's sensitivity to stress. The HPA axis, which includes the hypothalamus, pituitary, and adrenal glands, is thought to have a role in the body's reaction to stress. Stress may have a role in the etiology of infertility since HPA affects spermatogenesis.[2]

It has been hypothesized that chronic exposure to stressors, including high levels of psychological stress, might increase the body's production of ROS. Because of their high lipid content, spermatozoa are more likely to undergo lipid peroxidation when exposed to oxidative stress due to a lack of cytoplasmic enzymes capable of scavenging ROS. An individual human being may become sexually neurasthenic and functionally impotent due to the stress and pressure of contemporary life. This might cause a new wave of inferiority complexes among sexually active people, along with other related disorders. Poor quality semen has been documented even when male animals are kept in ideal surroundings and fed at the perfect times.[3-4]

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It is widely established that both in people and in experimental animals, alcohol consumption reduces fertility. Low sperm count and sperm motility are two of the most common reproductive issues observed in alcoholics. Testicular atrophy, or the decrease in the size of a man's testicles, is a result of heavy alcohol use. Alcohol consumption significantly increased lipid peroxidation in the testis and reduced the activities of 3-hydroxysteroid dehydrogenase and 17-hydroxysteroid dehydrogenase, thereby preventing the conversion of dehydro-epi-androsterone and andro-stenedione to testosterone the levels of glutathione peroxidase and superoxide dismutase were reduced in the testes of alcohol-treated rats, whereas malonodialdehyde production was significantly increased. To stimulate androgenesis in the testes, an antioxidant is required because ethanol causes oxidative stress, which results in the production of free radicals.[5]

2. Material and Methods

For the experiment, we used 30 male Wistar rats and randomly split them into 5 groups of 6. The categories are a regular control, a sterile control, a positive control, an experimental I group, and an experimental II group. Young rats in all four groups were rendered sterile through oral administration of 3 gm of ethanol / kg body weight / day for 4 weeks . As is standard practice, sterility was established through testicular biopsy, observation of sexual behavior, and study of testosterone hormone levels in all groups. Drugs were administered to each group when it was determined that alcohol use had resulted in sterility.

"For 30 days, two groups of rats were given either sterile water or an ethanolic extract of Cycas circinalis or Ionidium suffruticosum via oral gavage tube. The normal control and sterile control groups received sterile water, while the positive control received testosterone hormone subcutaneously."

- **Extraction Of Cycas Circinalis And Ionidium Suffruticosum**:

Botanical material gathering:

Following the procedures outlined in the introduction, male cones of Ccand Is were collected, authenticated by experts, and stored in a herbarium at the Pharmacology department for future identification purposes. "After being collected, cleaned with distilled water, and air dried for 10 days, the male cones of Cycas circinalis and the complete plant of Ionidium suffruticosum were used." Cc male cones and Is plants were dried and ground into a powder.

Methods for Making Raw Herbal Extract:

The powdered Cc and Is were used to begin the extraction process, which resulted in a crude plant extract. About 50 grams of plant material powder was extracted using the hot continuous percolation technique in a Soxhlet for 24 hours. The solvent used was ethanol, which has been standardized in our previous research. Since ethanol is a universal solvent, it was utilized to extract the plants. In this study, we used ethanol for extraction of both herbs since it conformed to the standards we had previously established for acceptable solvents in extraction. Most phytoconstituents may be dissolved in ethanol, making it a preferable solvent for extraction. When ethanolic extract was compared to extracts made with other solvents such methanol, chloroform, aqueous, and benzene, it was shown to have the highest concentration of phytoconstituents. After drying in a hot air oven, the ethanolic extracts of C.circinalis or I.suffruticosum were concentrated in a hot water bath. Cc extracts had a 7.14% yield, whereas Is extracts had an 8.42% yield.

- **Experimentalprotocolforstudyingthefertilityeffectof*c.circinalis*and*I.suffruticosum*inmalewistarrats:**

Drug administration and animal grouping in experiments:

Simple Random Sampling is used for sampling.

Table1: Male Wistar rat sex distributions for research

Groups	Group-A	Group-B	Group-C		
			C1	C2	C3

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Positivecontrol	6	6	6	6	6
Normalcontrol	6	6	6	6	6
C.circinalis(ExpI)	6	6	6	6	6
Sterilecontrol	-	-	6	6	6
I.suffruticosum(ExpII)	6	6	6	6	6

"Housed in polypropylene cages with rice husk bedding, standard rat pellets, and water available ad libitum, and acclimated to a 12/12 light/dark cycle at 23-25 °C, a total of 138 adult male Wistar rats were used in the study. Of these, 114 were young rats and 24 were old rats."

- **Sexual Behavior of Male Rats**

Breeding and preparing male rats:

Before delivering the medicines, sexually active male rats were chosen for testing and taught for sexual behavior twice daily for 10 days.

Female rats are prepared by:

Using benzoate oestradiol (10 pg/100 g) and progesterone (500 pg/100 g) administered subcutaneously, 48 hours prior to the copulatory studies, adult healthy young female rats of 8 weeks' age weighing about 130-140 gm were selected and brought into oestrous phase.

Research on rat copulation:

The test was conducted in a 50-cm-by-30-cm-by-30-cm box illuminated by a faint yellow light. Two female rats, marked with picric acid as described above, were placed in the box with one male rat picked at random from each group. Following an acclimation period of 15 minutes, the following aspects of sexual behavior were documented**:**

a. **Mount frequency:** The total number of mounts from the moment the female is introduced to the moment she ejaculates that do not have an intromission.

b. **Intromission frequency:** The total number of intromissions from the moment the female is introduced until she ejaculates.

c. **Mount latency:** The lag period between when the female is introduced and when the male initially mounts.

d. **Intromission latency:** The period that passes after a woman is introduced before a man makes his own introduction.

e. **Ejaculation latency:** The period that passes after a woman is introduced before a man makes his own introduction,

f. **Post-ejaculatory interval:** The period between one ejaculatory act and the beginning of the subsequent series,

g. **Total sexual behavior:** For an hour, male and female rats were monitored for behaviors such genital grooming, ano-genital smelling, and chasing until a female ejaculated.

- **Sample collection:**

Male rats were anesthetized with xylazine and ketamine after their sexual behavior was observed and were then put on a sterilized dissection board. The skin was incised and opened to a length of approximately 2 cm on the animal's ventral neck. The jugular vein being located, Gauze needle and a disposable 3 ml syringe being used to extract 2 ml of blood for testosterone hormone analysis.

Transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffered saline was performed on the left ventricle and the right atrium was opened to remove blood from the testicular blood vessels and replace it with buffered saline for proper fixation of the testes. When the animal's limbs twitch, its tissues blanch, and its whole body stiffens, it means that the perfusion was successful. The testes were extracted by a pre-scrotal incision created after perfusion.

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Incision of the tunica vaginalis revealed the testicles. There was a hole in the spermatic cord. With the scalpel, the epididymis was cut away from the testes. We used a Vernier caliper to determine testicular size followed by weighing and placing the testicles in Gendre's solution for fixation.

Gently squeezing and milking out the epididymis yielded the sperm that were then utilized arbitrarily for smear preparation and sperm count. Under a microscope, the collected sperm were analyzed for their viability and mobility after being placed on a glass slide with a cover slip. Both the control and experimental groups' motility and viability of spems were measured and compared numerically.

Taking a testicular circumference reading:

Testes were measured for their length, width, and height using a Vernier caliper and their weight was determined using a digital weighing machine after they were removed from the epididymis. Right and left testes were measured and weighed independently, and the averages were then calculated. Lambert's formula was used to determine testicular volumes.

$$(Volume = Length \times Breadth \times Height \times 0.71$$

Gonado-Somatic Index:

Testicular relative weight, or gonado-somatic index (GSI), was determined using the following formula.

$$GSI = Weight\ of\ testis\ in\ grams\ /\ Body\ Weight\ in\ g$$

GSI-Gonado Somatic Index

Weight of testis - Absolute weight of testis in gram
Body Weight -Weight of ratsonthe40thdayingram

A weighing machine was used to determine the rat weights. Rats were weighed 30 days following medication delivery and again 40 days later after a rest period. Differences in weight over 40 days were recorded, and that number was used to determine GSI. The GSI values were then tabulated after calculation.

- **Semen Analysis:**

Sperm count:

The cauda epididymis was milked out onto a glass slide, and the resulting sperm was stored at 37 degrees Celsius with a single drop of 2.9% sodium citrate (Oyeyemi et al., 2008). The epididymis from which the semen was taken was chosen at random. Semen smears were prepared from the opposite epididymis. In order to determine how many spermatozoa were present, an upgraded double Neubauer ruling Chamber (Depth 1/10 mm) was employed. In the WBC diluting pipette, the spermatozoa samples were diluted 1:10 with normal saline, and the diluent was drawn to the 0.5 mark. The sperms were counted under high power in the 4 WBC squares in the same way as the total leucocyte count, which included mixing the contents of the bulb for 5 minutes and discarding the initial few drops before charging the counting chamber with a cover slip over it.

Calculation for sperm count: N = the number of sperm in a 64x64 grid. $(Volume = 4/10\ mm^3)$

Morphological analysis of sperm:

Spermmotilityand viability:

Under a microscope, the motility and viability of 100 individual spermatozoa were observed, and a percentage was determined.

Preparationofsmear:

The spermatozoal samples were used to make smears. Three smears were made from spermatozoal samples collected from each rat. A glass slide was used to collect the sperm, and then one drop of 2.9% sodium citrate was mixed thoroughly with seminal fluid, and the spreader was brought back to contact the drop, causing it to spread along the border of the spreader. Quickly and uniformly, with mild but equal pressure, the spreader was moved to the left from the right side. The spermatozoal suspension moves in a film behind the spreader. Waving it in the air helped dry it off rapidly. Papanicolaou stain was used to color the slides.

Morphologyof Sperm Cells:

All smears were examined for morphological characteristics under oil immersion (100 X). Headlessness, rudimentary tails, curved midpieces, curved tails, looped tails, bending midpieces, tailless heads, and bent tails were all seen in both the control

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and experimental groups counting 50 sperm cells each smear and 3 smears per rat, a total of 900 sperms were tallied for each group, including both normal and aberrant sperms. Chi-square test was used to evaluate the data.

- **EstimationOfSerumTestosteroneLevel:**

After letting blood clot for 40 minutes, serum was extracted by centrifuging the blood at about 3000 rpm. Serum was extracted from blood samples using micropipettes.

Method of measuring Testosterone Levels:

After adding the conjugate (100 l) to each well, we added the serum samples (25 l) and mixed everything together. After the contents of the wells were removed and the wells were incubated at 37 °c for an hour, the wells were washed twice with 300 l of distilled water, 100 l of TMB-Substrate was added to each well, the wells were incubated for 15 minutes in the dark, and finally, 100 l of stop solution was added to each well. The ELISA analyzer was used to collect measurements once the preceding steps were completed . The numbers were recorded and tallied.

- **Histo-MorphometricAnalysis:**

Histomorphometric analysis was performed using Micro analyzer software after examining the dyed slides for histological alterations. Because seminiferous tubule are so complex, and because each tube is sliced by multiple times in every segment of the testis, focusing the stained slides required the use of 10x objectives. Six testicular sections (300 tubules) were collected from each animal, and each section included a random sample of 50 almost circular seminiferous tubules for counting and diameter. In the end, we tallied up the average diameter of each group's seminiferous tubules. The binocular lenses of the research microscope were used to concentrate on the stained slides. Micro analyzer software was used to count Leydig cells, sertoli cells, and spermatogenic cells at a magnification of 40X and to estimate the nuclear diameter of each cell. Leydig cells clustered around blood arteries in great numbers, and their cytoplasm included many cells with oval or spherical nuclei. Sertoli cells were seen to be rather large. Both the size and number of the nuclei of secondary spermatocytes are reduced from those of original spermatocytes. Two spermatids are produced from the division of a secondary spermatocyte. Each spermatid had a spherical nucleus and seemed to be a round cell. The cell and nucleus are noticeably smaller compared to those of spermatogonia and spermatocytes. Final mean nuclear diameters were measured and tabulated for leydig cells, sertoli cells, the spermatogonium, and primary and secondary spermatocytes.

- **Statistical Analysis:**

After collecting and tabulating the data, statistical analysis was performed. We tabulated our data after computing the mean, standard deviation, and standard error of the mean. We compared the two groups using the unpaired t-test, analyzed the data within each group using one-way ANOVA, and then compared the groups using the chi-square test and the post hoc pair wise comparison test for sperm morphology. SPSS was used for the statistical analysis, while Graph Pad Prism was used for the graphing.

3. Results

Alcohol-induced sterility in a cohort of young rats confirmed:

Table2: Alcohol-induced sterility in young rats: confirmatory criteria

Parameter	Mounting Index (MI)	Total sexual behavior (TSB)	Serum Testosterone Hormone (ng\ml)
Normal control (Mean ± SEM)	6.95 ±1.68	202 ± 0.967	2.1 ± 0.21

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Sterile rats (Mean ± SEM)	4.55 ± 1.10#	134 ± 0.61***	1.2 ± 0.12**
T	1.19	59.47	3.72
P value	0.26	0.001	0.01

All metrics except mounting index were determined to be statistically significant in the comparison between control and sterile rats. "Compared to normal rats, which showed no histopathological changes in the tubules, alcohol-treated rats showed degeneration of seminiferous tubules with devoid of germ cells, increased luminal diameter, desquamation of the epithelium in the tubules, and only a few germ cells attached to the basal lamina of the tubules." Sterile rats were shown to have lower levels of both sexual behavior and testosterone compared to their healthy counterparts. This demonstrates that alcohol administration led to the induction of sterility in experimental rats.

Alcohol-induced sterile rats: C.circinalis and I.suffruticosum's effects on fertility:

Table 3: Alcohol-induced sterility in rats: fertility and implantation rates (Group C1)

Parameters	**MI**	**TSB**	**BW (gm)**	**VT (cu.cm)**	**WT (gm)**	**GSI**	**SC (millions/ml)**	**TH (ng/ml)**
NC	6.58 ± 1.80	186.20 ± 0.7	181.17± 1.9	0.83± 0.05	0.95 ± 0.01	0.48 ± 0.01	30.86 ± 0.40	2.26 ± 0.05
SC	5.83±0.31	133.2±3.2	159.7±3.4	0.61±0.03	0.73±0.03	0.46±0.02	25.55±1.4	1.37±0.08
PC	8.20±0.37	195±3.85	208.3±4.7	0.89±0.13	1.12±0.06	0.54±0.02	57.31±2.1	4.02±0.22
E I (Cc)	7.17±0.24	193±1.69	194.8±1.6	0.84±0.01	1.04±0.05	0.53±0.25	43.26±1.6	2.87±0.12
E II (Is)	7.92±0.13*	199.8±1.5 *	208±3.7*	1.28±0.09 *	1.18±0.05 *	0.56±0.02*	54.3±2.16 *	3.48±0.14 *
F	15.2	134.5	39.01	22.96	16.22	4.65	78.14	58.85
P value	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001

Table 4: Alcohol-induced sterility in rats: testicular morphometry (GroupC1)

Parameters	**EH (µm)**	**LCN dm (µm)**	**STdm (µm)**	**SCN dm (µm)**	**SN dm (µm)**	**PSN dm(µm)**	**SSN dm (µm)**
NC	82.50±2.19	5.03±0.07	258.6 ± 3.2	7.08±0.08	4.9±0.17	7.22±0.04	5.43±0.09
SC	78.67±1.52	3.9±0.13	222.3±5.01	5.35±0.06	4.48±0.09	6.10±0.09	3.5±0.08
PC	99.8±1.97	7.18±0.12	276±5.7	8.70±0.24	5.67±0.19	8.12±0.13	5.43±0.11

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EI (Cc)	105.2±1.6	7.35±0.14	281.2±5.4	7.75±0.07	5.3±0.09	7.53±0.08	6.33±0.09
EII (Is)	114.7±2.1	8.35±0.07	303±1.24	8.58±0.11	6.12±0.07	8.6±0.09	7.43±0.09
F	64.76*	290.35*	46.07*	117.59*	22.95*	112.8*	223.25*
P Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Randomly chosen 30 circular sections of seminiferous tubules were examined for the presence of spermatogonium and spermatocytes. Spermatogonium and spermatocyte counts were averaged. Mean standard error of the mean is used to display the data. Spermatogonium (64.2 ± 1.9) numbers were significantly lower in normal control than PC (86.2 ± 2.2), EII (81.2 ± 2.1), and EI (78.6 ± 21.9). When comparing the control group (106.2 4.8) to the sterile control group (42.4 ± 1.2), there was a significant difference between the PC group (178 ± 5.2) and the EII group (172.2 ± 5.1) and the EI (164.2 ± 4.2). The number of spermatocytes in the sterile control group was significantly lower than in the normal control group (106.2 ± 4.8) (P< .0001). One-way analysis of variance was used to examine the data, and the results showed statistical significance (P< 0.05). Histomorphometric analysis of the testes revealed that EII had larger nuclei than the control groups.

Table 5: Sperm morphology of sterile rats

Sperm Morphology	Abnormal Sperm	Normal Sperm	Total
NC	240	660	900
SC	336	564	900
PC	158	742	900
EI (Cc)	164	736	900
EII (Is)	118	728	900
Total	1016	3483	4500

The groups were compared based on their sperm's viability and mobility. Sperm motility, which is typically 46.2% in controls, is elevated to 83.2% in PC, 82.4% in EI, and 86.5% in EII. The motility of sperm in the sterile control group was lower (39.4%) than in the normal control group (46.2%). The number of defective sperm significantly decreased in the experimental groups. Chi-square analysis of the data confirmed their significance.

Table 6: Post Comparison of alcohol-induced sterile rats on a pair-by-pair basis

Parameters	BW	MI	WT	TSB	SC	VT	SCN dm	GSI	LCN dm	TH	SN dm	EH	SSN dm	ST dm	PSN dm
NC / SC	S	n/s	S	S	n/s	S	S	n/s	S	S	n/s	n/s	S	S	S
NC/ EII	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

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NC /PC	S	S	n/s	n/s	S	n/s	S	n/s	S	S	S	S	n/s	n/s	S
NC/EI	n/s	n/s	n/s	n/s	S	n/s	S	n/s	S	S	n/s	S	S	S	n/s
SC/EII	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SC/ PC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PC/ EII	n/s	n/s	n/s	n/s	n/s	S	S	n/s	S	n/s	n/s	S	S	S	S
SC/EI	S	S	S	S	S	S	S	n/s	S	S	S	S	S	S	S
PC/ EI	n/s	n/s	n/s	n/s	S	n/s	n/s	n/s	n/s	S	n/s	n/s	S	n/s	S
EI/EII	n/s	n/s	n/s	n/s	S	S	S	n/s	S	S	S	S	S	S	S

One-way analysis of variance showed that all of the parameters were statistically significant at the P< 0.001 level. Post hoc pairwise analysis reveals statistical significance only between NV and EII, SC and EI, SC and EII, and SC and PC, but not between any of the other groups.

4. Discussion

In humans, abnormalities in spermatogenesis have been linked to long-term alcohol usage. Male drinkers have lower testosterone levels, which may lead to erectile dysfunction and infertility. According to research, ethanol is one of the most toxic substances to the testicles. Testicular apoptosis was induced by ethanol treatment, which was followed by testicular DNA fragmentation and an increase in the number of apoptotic spermatogonia and spermatocytes.[6] The secretory activity of sertoli cells is inhibited by alcohol.[7]Atrophy of the testes results from a decline in sperm production and the size of the seminiferous tubules. Desquamation of the seminiferous tubules and significant alterations in the nuclear diameter of the germinal epithelium were seen in the current investigation. In rats, alcohol use led to testicular lesions characterized by a reduction in the diameter of the seminiferous tubules and a decrease in the number of Leydig cells.[8-9]While in mice, alcohol consumption led to degenerative alterations of the epithelial component of the seminiferous tubules.[10]

When compared to sterile control rats, testosterone, Cc, and Is-infused animals exhibited much higher sexual activity. Changes in testicular weight, sperm count, sperm motility, and sperm morphology were seen in sterile rats after alcohol exposure. Body weight, testicular weight, and GSI, testosterone hormone, were all higher in experimental rats than in sterile rats. Nearly twice as many sperm were counted as in sterile controls.

5. Conclusion

Testicular tissue from alcohol-treated rats was found to have larger-than-normal multinucleated cells in the tubules and empty interstitial spaces compared to control rats Rats given Cc and Is had normal tubular epithelium, an increased quantity of spermatids in the tubule lumen, and spermatogenic cells at several stages. Alcohol-induced sterile rats had a smaller seminiferous tubule diameter and a shorter germinal epithelial height. In addition, alcoholic mice had thinner basal lamina and tunica albuginea of the seminiferous tubules than their control counterparts.

C.ciricnalis and I.suffruticosum's steroidogenic action may be responsible for restoring fertility. Experimental groups with Cc and Is administration revealed normalization of ethanol-induced testicular dysfunction, as shown by an increase in sperm count, a reduction in aberrant spermatozoa, and an increase in testicular weight.

To date, there has been just one published scientific study on the reproductive impact of Cc and Is, which examined its effects on alcohol-induced sterility in male Wistar rats. Future research will focus on pinpointing the phytoconstituents that trigger steroidogenesis and the androgenic impact they have.

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