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Research Article

Testicular Zinc, Sperm Parameters and Redox Balance in Ethanol-Induced Reproductive Dysfunction Treated With D-Ribose-L-Cysteine

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Abstract— This study explores the impact of alcohol, a widely abused substance, on the reproductive system, particularly testicular function. The investigation introduces the question of whether concurrent administration of D-Ribose-L-Cysteine supplement (Cellgevity) can mitigate the detrimental effects of alcohol on the testes. The primary objectives involve assessing the influence of D-Ribose-L-Cysteine on testicular zinc levels, spermatogenesis, and redox balance. The experiment employed 20 Sprague Dawley rats, distributed into 4 groups, each consisting of 5 rats: a control group without ethanol or cellgevity, an Alcohol group subjected to ethanol induction only, a Cellgevity group receiving cellgevity only, and an Alcohol plus Cellgevity group exposed to ethanol while concurrently treated with cellgevity. The study duration was 14 days for each group. Results revealed noteworthy alterations in oxidative stress markers. The Alcohol group exhibited a statistical decrease in Superoxide Dismutase (SOD), Catalase (CAT), Glutathione (GSH), and an increase in Malondialdehyde (MDA) and serum zinc concentration. Conversely, the Alcohol plus Cellgevity group demonstrated a statistical increase in SOD, CAT, GSH, and a decrease in MDA and serum zinc concentration. The study also explored sperm parameters, indicating a statistical decrease in sperm motility and morphology in the Alcohol group compared to the Alcohol plus Cellgevity group. However, sperm concentration remained unaffected. In conclusion, ethanol administration adversely affects various aspects of male reproductive functions, including sperm motility, morphology, and testosterone levels, alongside disturbances in trace elements within the testes and increased oxidative stress. Notably, D-Ribose-L-Cysteine administration, as represented by Cellgevity, exhibited a beneficial effect on male reproductive functions. It mitigated oxidative stress, prevented a significant reduction in testicular zinc levels even after ethanol induction, and presented a potential avenue for safeguarding male reproductive health in the face of alcohol-induced stressors.

Keywords— Alcohol, D-Ribose-L-Cysteine, Reproductive system, Spermatogenesis, Oxidative stress, Testicular zinc, Sprague-Dawley rats.

1. Introduction

The reproductive system is extremely important for procreation, so its importance in the modern world cannot be understated. It has however been observed that the male reproductive system is under impending attack which can be due to a variety of environmental substances, one of which is thought to be alcohol, which can be harmful to the

reproductive system. Alcohol use and its impact on testicular function are, in fact, rarely discussed these days. All parts of the male reproductive system have been demonstrated to be negatively impacted by alcohol, with the testes, anterior pituitary gland, liver, and hypothalamus among its targets [1]. Long-term alcohol consumption in lab animals has been linked to substantial degenerative alterations including damage to germ cells, atrophy of the testicles, loss of minerals, and a decrease of the seminiferous tubules [2]. In addition, it has been documented that individuals with a history of long-term alcohol misuse experience spermatogenic failure, hypogonadism, gonadal dysfunction, and infertility due to testicular germ cell death. The testicular Sertoli cells' secretory activity is likewise negatively impacted by ethanol exposure [3], and the blood-testes barrier mediated by the Sertoli cells is compromised [4]. Since Sertoli cells are the major target of ethanol toxicity, ethanol-induced testicular injury can be identified morphologically by the significant vacuolization of Sertoli cells [5]. Consuming alcohol causes apoptotic cell death in a variety of cell lines and organs, such as the testis, thymus, liver, and brain [6]. Oxidative stress caused by free radicals is the most common alcohol-induced dysfunction that endangers the health of the reproductive system, particularly testicular functions. One of the synthesized antioxidants that helps cells make glutathione when needed is the D-Ribose-L-Cysteine supplement (Cellgevity^{TM}). Consuming glutathione in its whole form is ineffective since the gastrointestinal digestive process would degrade it before it could enter the cell. These challenges are resolved by the ribose component of Riboceine (the active ingredient of D-Ribose-L-Cysteine), which efficiently transports and protects the delicate and susceptible cysteine molecule, allowing cells to synthesize glutathione as needed [7]. L-cysteine is a precursor of glutathione, which is thought to be crucial for the detoxifying of oxidative stress in cells [8]. L-cysteine is a semi-essential amino acid, meaning that the human body can synthesize it from the amino acid methionine along with a variety of proteins [8,9]. Since D-ribose-L-Cysteine has effective antioxidant properties and reduces the harmful effects of aluminum chloride on the testes, it can increase male fertility [10]. Rats' oxidative stress and insulin resistance brought on by sucrose are reduced when they consume whey protein and alpha-lactoalbumin, two proteins high in cysteine [11]. The capacity of D-ribose-L-Cysteine to enhance and improve testicular zinc, spermatogenesis, and redox balance in male rats with ethanol-induced reproductive failure is the main emphasis of this work.

1.1 Significance of Study

The present study is of utmost importance in the world of science as it explores the possible protective effects of D-Ribose-L-Cysteine supplement against long-term alcohol-induced impairments in male fertility. Our knowledge of the complex physiological and molecular processes underlying the interactions between alcohol, oxidative stress, and male reproductive health will be further advanced by the findings of this study. The results could also aid in the creation of supplements and focused therapy interventions meant to protect sperm parameters and testicular function from alcohol-induced stresses. The work is in line with a translational research strategy, connecting basic knowledge with possible therapeutic uses and adding to the larger body of knowledge on reproductive biology and the effects of drug usage.

1.2 Aim of Study

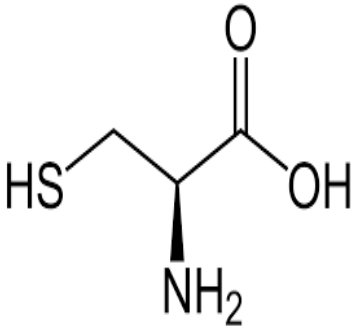
In male Sprague-Dawley rats with ethanol-induced reproductive failure, the aim of this study is to examine the protective effects of D-ribose-L-Cysteine supplementation on sperm parameters, testicular zinc, and redox balance.

1.3 Objectives of the Study

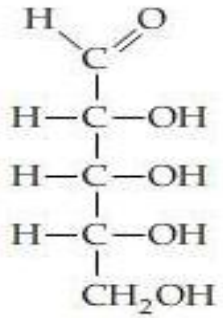
1. To evaluate the effect of ethanol-induced reproductive damage on testicular zinc and sperm parameters.
2. 2. To understand how redox balance is affected by ethanol-induced reproductive damage
3. 3. To determine whether D-Ribose-L-Cysteine may be beneficial for objectives (1) and (2).

2. Related Work

2.1 D-Ribose-L-Cysteine's impact on male reproductive processes



L-cysteine



D-ribose

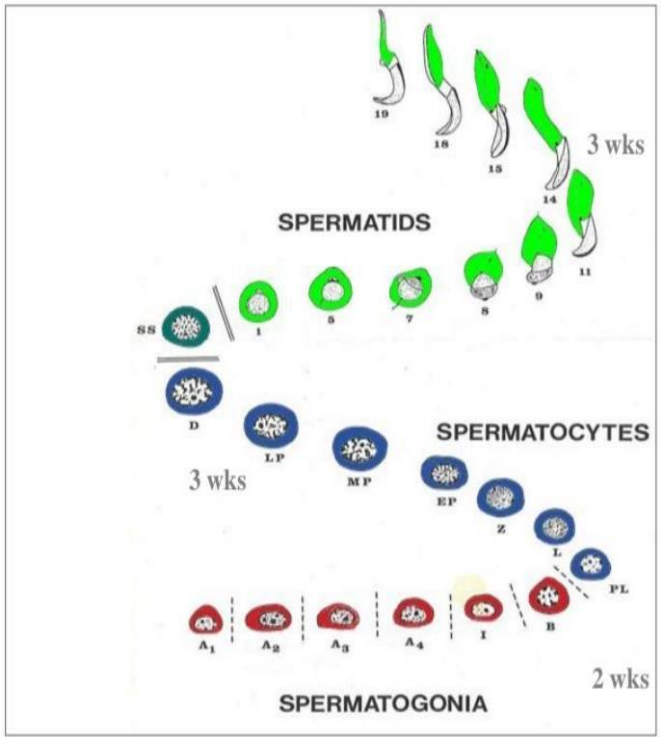
D-Ribose-L-cysteine supplement (cellgevity) may have some effects on reducing oxidative stress levels in organs, including the testes, according to recent research [10]. It has been documented that D-Ribose-L-Cysteine (DRLC) protects the testis from radiation and enhances fertility by preserving the structural and functional integrity of the testicles [12]. Due to its strong antioxidant properties, D-Ribose-L-Cysteine can increase male fertility by lessening the harmful effects of aluminum chloride on the testes [10]. As a result, D-Ribose-L-cysteine supplementation ought to supply adequate antioxidants that can shield animals' testicles from the damaging effects of reactive oxygen species and maintain their structural and functional integrity.

2.2 Spermatogenesis in Rats

The process known as spermatogenesis produces highly differentiated, haploid spermatozoa (sperm) from primitive, diploid stem cell spermatogonia. This is a complicated process because it involves spermatogonial stem cells next to the seminiferous tubule basal membrane of the testis dividing, replenishing the stem cell population, and producing progeny cells that concurrently develop into spermatozoa [13]. In the 10–20 seminiferous tubules that make up each rat testis,

spermatogenesis takes place. The somatic Sertoli cells that border the seminiferous tubules and support and nourish germ cells during spermatogenesis control the process [14].

Spermatogenesis is the result of a sequence of mitotic divisions of spermatogonia, the last of which produces spermatocytes. The cell that goes through the protracted process of meiosis, known as spermatocyte, is the one that duplicates its DNA during preleptotene, pairs and condenses its chromosomes during pachytene, and ultimately completes two reductive divisions to create the haploid spermatid. The spermatid starts off as a straightforward round cell but quickly goes through several intricate morphological modifications. A whip-like tail enclosing a flagellum and densely packed mitochondria are formed by the nuclear DNA, which is extremely condensed and stretched into a head region coated by a glycoprotein acrosome coat. The rat spermatogenic cycle's stages can be distinguished by the progressive morphological steps in spermatid development.



2.3 Role Zinc (Zn) In Spermatogenesis

The second-most essential trace element, zinc (Zn), is widely distributed throughout the human body. More than 200 metalloenzymes depend on zinc as a micronutrient to function [15].

According to Mirnamniha [16], it performs several tasks during the pre-fertilization process, including sperm capacitation, binding of the sperm zona pellucida, the acrosome reaction process, penetration at the zona pellucida site, participation in the sperm and oocyte binding process, activation of the egg or zygote, and the final zona reaction. Additionally, it is necessary for the stability of the cell membrane and the upkeep of germ cells. Because zinc regulates spermatogonia proliferation and is necessary to maintain germ cells unharmed during the meiotic phase, its content in the testis rises during the early stages of spermatogenesis [17].

Spermatogenesis is significantly impacted by zinc finger proteins (ZFPs) [18]. Numerous scientists have talked about how important ZFPs are for both spermatogenesis and the growth and differentiation of germ cells. According to Sun [19], ZFP185 overexpression in Leydig cells promotes the generation of testosterone and plays a significant role in spermatogenesis.

Spermatogenesis is significantly influenced by zinc transport proteins (ZIPs), which function at the intracellular zinc level and facilitate zinc uptake into the cytoplasm [20]. At various phases of spermatogenesis, decreased expression of ZIPs during the transport and uptake of zinc into the intracellular lumen may result in defects [21]. Zinc consumption levels during spermatogenesis and circulating levels are positively correlated [22].

Rat testes with low zinc exhibit decreased levels of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein activity. According to earlier research, the testes contain a high quantity of zinc, and deficiencies in zinc hinder spermatogenesis and result in defective sperm. Zinc insufficiency can lower serum testosterone levels and hinder spermatogenesis [23]. Idiopathic male infertility and low sperm quality are caused by a low zinc content in the diet. In the sperm head and tail, it preserves genomic integrity [24].

2.4 Effect of Ethanol on Spermatogenesis and Testicular Zinc

According to Babakhanzadeh, et al [25], a significant proportion of male-related infertility cases consume alcohol. This suggests that alcohol use is a strong risk factor for male factor infertility. Additionally, studies have shown a link between consuming alcohol and lower testosterone levels [26]. The anterior pituitary gland's response to alcohol was a reduction in LH and FSH output. This result is consistent with the findings of Weera and Gilpin [1], who reported that alcohol not only interferes with the production of LH and FSH but also with their secretion and the secretion of stress hormones.

Long-term alcohol consumption in experimental animals has been linked to notable degenerative changes like testicular shrinkage, damage to germ cells, and a decrease in the size of seminiferous tubules [3, 5]. Furthermore, people with a history of chronic alcohol misuse, spermatogenic failure, hypogonadism, gonadal dysfunction, and infertility have been observed by inducing apoptosis in testicular germ cells [27]. Consumption of alcohol causes apoptotic cell death in a variety of cell types and organs, including the brain, liver, thymus, and testis [6, 28].

2.5 Antioxidant Systems and Oxidative Stress in the Testes

The highly vigorous replicative process known as spermatogenesis can produce up to 1,000 sperm per second.

High rates of mitochondrial oxygen consumption by the germinal epithelium are implied by the high rates of cell division intrinsic to this process. However, the testes have inadequate vascularization, which results in low oxygen tensions and strong competition within the testes for this vital element [29].

The testicular microenvironment has moderate oxygen tensions, but because of the high content of highly unsaturated fatty acids and the existence of systems that have the ability to produce reactive oxygen species (ROS), this tissue is nonetheless susceptible to oxidative stress. The mitochondria and several other enzymes, such as the xanthine- and NADPH-oxidases [30, 31] and the cytochrome P450s [32], can produce reactive oxygen species (ROS). These enzymes either intentionally create ROS or unintentionally create these harmful molecules as a byproduct of their metabolic activity. The testes have created a complex network of antioxidant systems to counteract this risk. These systems include both non-enzymatic components (such as transition metal-binding proteins) and enzymatic components (such as catalase, glutathione reductase, and superoxide dismutase) and lipid phase chain-breaking antioxidants.

This intricate antioxidant defense mechanism was created to protect the testis from oxidative stress and injury. They have a critical role in preventing lipid peroxidation. Different isoforms of glutathione peroxidases (GPx) can catalyze the reduction of peroxides through using reduced glutathione (GSH) as a reducing power [33]. Highly expressed in both spermatogenic and Leydig cells, the phospholipid hydroperoxide GPx (PHGPx) is one of the most significant GPx isoforms in a testicular environment [34].

2.6 Impact of Ethanol on Redox Balance in Male Reproductive System

The well-known oxidative stress that ethanol causes in the testes is extensively documented [5, 35]. According to reports, oxidative stress, testosterone suppression, and DNA damage are the major mechanisms by which ethanol-induced testicular damage is mediated [36]. Alcohol has an impact on how mitochondria work. The ATP that mitochondria make is necessary for sperm cells' flagella to move. Therefore, decreased or compromised mitochondrial function will hinder the motility of sperm. Alcohol is known to worsen mitochondrial function by elevating oxidative stress, which suggests that its impact on mitochondrial function is linked to the way it reduces sperm motility.

Alcohol-induced tissue injury is widely recognized to be caused by increased oxidative stress, especially in the liver, heart, central nervous system, and testis [37, 38]. Oxidative stress causes apoptosis breaks down DNA, and impairs reproductive function in the testis, which can result in abnormalities in embryonic development or fertility [39, 40].

Research indicates that both acute and long-term alcohol administration led to an increase in lipid peroxidation. This further suggests that the mechanism by which alcohol causes testicular toxicity involves elevated levels of free radicals or reactive oxygen species as well as lipid peroxidation [35, 41].

2.7 Lipid Peroxidation by Ethanol Induced-Oxidative Stress

Lipid peroxidation in the cell membrane can disrupt the fluidity and permeability of cell membranes and damage all cells. In other words, when free radicals damage the cell membranes, their protective cell is lost and thus the total cell is exposed to risk. In this regard, increased production of ROS induces lipid peroxidation in spermatozoa, which has two important effects: 1) Reducing sperm combination with oocyte; and 2) Increasing spermatozoa ability to bind to the transparent area (Zona Placida) [42].

Lipid peroxidation also resulted in abnormalities in the sperm's middle part and decreased fertilization potential of the acrosome. Malondialdehyde (MDA) molecules a by-product of lipid peroxidation permeates the cell membrane structure, resulting in an asymmetric distribution of lipid membrane components. Remarkably, the product of the secondary failure of primary lipid hydroperoxides is used to calculate the rate of lipid peroxidation [42]. The breakdown of the unsaturated fatty acid peroxides results in the production of MDA. Since it is an end product of lipid peroxidation, it is utilized as a marker, or biomarker, to ascertain the rate at which biotic and abiotic stress causes oxidative damage to lipids. Remarkably, the primary cause of testicular dysfunction at the moment is damage resulting from lipid peroxidation [43].

2.8 Damage to Sperm Morphological Characteristics by Ethanol Induced-Oxidative Stress

Since free radicals can greatly affect sperm characteristics like count, motility, and morphology, they can also lower sperm fertility [44]. Oxidative stress caused by free radicals plays a major role in the production and growth of aberrant sperm as well as in reducing sperm count and transformation and breaking down of sperm DNA. Infertility is caused by these sperm DNA alterations. In line with this, spermatozoa cultured at high oxygen pressures exhibit decreased sperm motility and rate; nonetheless, this impact can be avoided by including catalase into the culture medium [45]. Sperm motility may be decreased as a result of spermatozoa producing more hydrogen peroxide (H_2O_2) when exposed to high oxygen pressure [46].

2.9 Damage to DNA Sperm by Ethanol Induced-Oxidative Stress

The degradation of DNA increases with oxidative stress [47]. In addition, research suggests that the high concentration of ROS in sperm is the cause of DNA fragmentation, which is frequently observed in the spermatozoa of infertile individuals [42]. DNA samples from individuals with sperm azoospermia were found to have a higher rate of damage than samples from healthy individuals in a study on teratozoospermia patients. Furthermore, the amount of ROS produced by these sperm was shown to be the primary cause of this damage, which may be the reason for infertility in individuals with sperm azoospermia. Consequently, it is

thought that the primary cause of infertility is either an excess of reactive oxygen species (ROS) or a reduction in the antioxidant capacity of semen, which leads to oxidative stress conditions and, in turn, a decrease in sperm motility, an increase in sperm mortality, and DNA fragmentation [42].

Theoretically, we hypothesized that prolonged exposure to ethanol would cause a notable imbalance in oxidative stress markers, which would impair sperm parameters and testicular function. It is anticipated that concurrent D-Ribose-L-Cysteine delivery will lessen these effects and maintain testicular zinc levels, sperm quality, and redox balance.

3. Experimental Method/Procedure/Design

3.1 Experimental Animal

20 male Sprague Dawley rats were used in this study. These rats were acquired from the University of Lagos' College of Medicine's Animal Laboratory Center. They weighed between 50 and 90g, were about seven weeks old, and were house bred. The rats were housed in wire-mesh cages at the animal room of the University of Lagos' Department of Anatomy. They were kept at room temperature and on a 12-hour light-dark cycle. International norms for the use of animals in research were adhered to in every process. With protocol number CM/HREC/010/16/056, the study was approved by the Departmental Ethics Committee and the Health Research Ethics Committee on Animal Use, College of Medicine, University of Lagos, Nigeria.

After two weeks of acclimatization, the male Sprague Dawley rats were randomly assigned into different four groups (A–D), with each group consisting of five rats. Since these rats were quite young and weighed below 100g, they were force-fed to increase their weight and adaptability to the daily routine experimental procedures. After they had attained a weight of about 120-150g, oral administration (i.e. ethanol and Cellgevity^{TM} induction) began. Absolute ethanol administration of 70% water dilution (30% v/v of alcohol) was administered (with a concentration of 1.5mg/kg of diluted ethanol). The cellgevity was administered based on Human Dose Equivalent (HDE), 150 mg/kg body weight of cellgevity. Oral administration lasted for 14 days.

3.3 ANIMAL GROUPING

The rats were split up into four groups in four cages, with five rats per cage. The weight of each rat served as the basis for this categorization. The rats in Group A weighed the most. Group B weighed more than Group C and was the next in line. Group C weighed more than Group D.

Group A (Alcohol group)
Group B (Alcohol + Cellgevity group)
Group C (Cellgevity group)
Group D (Control group)

3.4 DRUG ADMINISTRATION AND ROUTE OF ADMINISTRATION

1. The First group (Alcohol group): This group was administered ethanol orally using an oral cannula for fourteen (14) days. Their weights were determined and recorded weekly.
2. The Second group (Alcohol + Cellgevity group): This group was administered ethanol and cellgevity. These administrations were done orally using an oral cannula for fourteen (14) days. Weekly weights were determined and recorded.
3. The Third group (Cellgevity group): This group was administered cellgevity group only using an oral cannula. This administration was carried out for fourteen (14) days and weekly weights were determined and recorded.
4. The Fourth group (Control group): No drug was administered to this group. However, their weight was determined and recorded weekly.

3.5 ANIMAL EUTHANASIA, BLOOD COLLECTION, AND HARVEST OF ORGAN

The rats were made to rotate in the air to allow for unconsciousness and immobility. Then, they were sacrificed using cervical dislocation. After which, each animal was dissected using the dissecting tools. The testes (left and right) and the epididymides (left and right) were harvested. The brains were also harvested and excised at the midpoint. These harvested organs were wrapped using filter papers to remove blood fluid and fat bound to the organs. Using an electronic sensitive weighing balance, the weight values of these organs were recorded. The harvested organs were kept in a plain sample bottle containing phosphate buffer. These sample bottles were placed on ice and each bottle was labeled according to each rat in the various groups.

Blood samples were collected from each rat in each group. The blood sample of each animal was collected through their eyes (via ocular puncture of orbital vein) with the capillary tube and dropped into a plain sample bottle each labeled according to each rat in each group. The plain sample bottles containing the blood samples were placed in a centrifuging machine and centrifuged at 300 revolutions per minute (rpm) for 20 minutes to separate blood components. After 15 minutes of centrifugation, the blood serum (supernatant) was gently extracted from each sample bottle using a syringe. The supernatant (serum) was placed in a different set of plain sample bottles having the corresponding labeling with the sample bottles that were centrifuged. The left testis and left epididymis were homogenized for oxidative stress analysis.

3.6 DETERMINATION OF OXIDATIVE STRESS (REDOX BALANCE) MARKER

The lipid and lipoprotein assay was done using the UV Spectrophotometer.

This parameter was observed and used as serum (supernatant) indices of oxidative stress during the analysis of the subjects:

- Malondialdehyde (MDA)

MALONDIALDEHYDE CONCENTRATION (MDA)

MDA is a routinely used biomarker to quantify the amount of oxidative stress and lipid peroxidation present in cells and tissues. Elevated levels of MDA are associated with multiple clinical disorders and a sign of heightened oxidative damage. MDA level was evaluated using the thiobarbituric acid

reactive substance (TBARS). 1.0ml of the supernatant was mixed with 2ml of TCA-TBA-HCL (tricarboxylic acid-thiobarbituric acid-hydrochloric acid) in a 1:1:1 ratio to measure the MDA level. The reagent was centrifuged for 10 minutes at 300 rpm after being boiled for 15 minutes at 100oC and allowed to cool. After removing the resultant, the absorbance at 532 nm was measured in comparison to a blank. The level of MDA activity is shown by the molar extinction coefficient for the MDATBA complex, which is 1.56x 105 $M^{-1}.CM^{-1}$ (i.e$\sum$ = 1.56x 105 $M^{-1}.CM^{-1}$.).

3.7 DETERMINATION OF ANTIOXIDANT ENZYMES LEVEL

The activity of the following antioxidant enzymes was determined spectrometrically to perform the antioxidant enzymes assay:

- Superoxide dismutase (SOD)
- Glutathione peroxidase (GPx)
- Catalase (CAT)

SUPEROXIDE DISMUTASE (SOD)

The ability of SOD to neutralize superoxide radicals and prevent the auto-oxidation of epinephrine was used to measure SOD activity. To find this, 0.02 ml of epinephrine in 0.005N HCl was introduced to a test tube that held 2.95 ml of 0.5 M sodium carbonate buffer, pH 10.2. This is a representation of the test sample. A reaction mixture of 2.95 milliliters of buffer, 0.03 milliliters of epinephrine, and 0.02 milliliters of water made up the standard sample. The inhibition of epinephrine auto-oxidation by SOD served as evidence of its presence. The amount of SOD activity was determined by measuring the change in absorbance at 480 nm for 5 minutes (i.e. an increase in absorbance as stated by Sun and Zigma in 1978).

GLUTATHIONE (GSH)

The reduced glutathione (GSH) content of homogenized testicular tissue was estimated using the Sedlak and Lindsay 1968 method for non-protein sulphydryls. The homogenized samples were centrifuged after 10% TCA (tricarboxylic acid) was added. Using 0.5 ml of Ellman's reagent (19.8 mg of 5,5-dithiobis-2-nitrobenzoic acid, or DTNB) in 100 ml of 0.1% sodium nitrate and 3.0 ml of phosphate buffer (0.2 M, pH 8.0), 1.0 ml of the supernatant was subjected to treatment. The reaction mixture was given a set amount of time (usually 5–10 minutes) to incubate at room temperature. This makes it possible for the yellow-colored product that is produced when DTNB and GSH react. The tissue sample's reduced glutathione (GSH) content was determined by measuring the absorbance at 412 nm.

CATALASE (CAT)

The Aebi method of 1984 was used in determining Catalase activity. 1.8 ml of 3.0 mM H_2O_2 was prepared with phosphate buffer to make up the reaction mixture. The phosphate buffer's baseline absorbance was determined with a spectrophotometer set at 240 nm. The absorbance is the blank absorbance. 0.2 ml of the tissue homogenate was mixed with this solution. This starts the catalase reaction, which breaks down H_2O_2 in the sample due to the presence of catalase. The reaction mixture is left to incubate at room temperature for 1 minute. To stop the reaction, acetic acid and potassium dichromate were added. For 1 minute, the absorbance of the reaction mixture was measured at 240 nm at 30-second intervals. The catalase activity was calculated using an extinction coefficient of H_2O_2 for 40.00 $M^{-1}.CM^{-1}$ (i.e.$\sum$ = 40.00 $M^{-1}.CM^{-1}$). The unit of measurement was moles per minute per mg of protein.

3.8 DETERMINATION OF TESTICULAR ZINC

Using a zinc colorimetric testing kit (Biovision, CA, USA) and the manufacturer's instructions, the testicular zinc level was determined. After the testicular tissue samples were homogenized, 50 µl of the 7% trichloroacetic acid (TCA) solution was added to 50 µl of the sample to briefly deproteinize it. After that, this was centrifuged for five minutes at 3000 rpm. A further step involved adding 20 µl of the plate well samples. Each standard and sample was then mixed with 200 µl of the zinc reaction mix, allowed to sit at room temperature for 10 minutes, and then the microplate reader was used to read the results at 560 nm. The amount of zinc present determines how intense the color is.

3.9 SEMEN ANALYSIS

The right testis and right caudal epididymis were removed for sperm analysis after the procedure of euthanasia.

Sperm concentration: Following the recovery of the testicles and caudal epididymis, 0.1 ml of epididymal fluid was added to 1.9 ml of water to create a 1:20 epididymal fluid ratio. After fully mixing the dilution, the sperm were counted using an improved Neubauer hemocytometer and a Leica D750 microscope (operating at a low magnification) to check that the sperm cells were evenly distributed to ensure accurate counting. Spermatozoa were counted within five red blood cell squares, including those that crossed the outermost lines on the top and right sides; those on the bottom and left sides were not counted. The quantity of spermatozoa counted was then represented in millions/ml.

$$Sperm\ concentration\ (million/ml) = Total\ counted\ sperm\ /\ Number\ of\ counted\ squares \times Dilution\ factor.$$

Sperm motility: This was carried out right away following the semen extraction. A drop of epididymal fluid was applied on a glass slide so that the spermatozoa were evenly dispersed. The slide was then covered with a 22 × 22 mm coverslip and inspected under a light microscope at a magnification of x100, with multiple fields being assessed. This process was used to measure motility. Next, three categories for sperm motility were established: progressive motile, non-progressive motile, and immotile. Using the subjective motility assessment, the various microscopic fields were evaluated, and the relative percentage of motile sperm was calculated and reported to the closest 5%.

3.10 STATISTICAL ANALYSIS

Data were analyzed statistically for serum testosterone, testicular testosterone, serum estradiol, testicular estradiol, sperm concentration, sperm motility, serum zinc, and

testicular zinc. When a one-way ANOVA indicated significant group differences, the data were presented as the mean and standard error of the mean (SEM). The particular group pairs that were statistically different were identified using Turkey's post hoc test. For statistical significance, a criterion of $P < 0.5$ was used. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for the analysis and used to determine the statistical significance of the data.

4. Results

Lipid Peroxidation and Antioxidant Enzymes Activities: The beneficial effect of D-Ribose-L-Cysteine on oxidative stress biomarkers and antioxidant enzyme activities were observed in both the testis and epididymis.

The figures below show the experimental illustration of these findings.

4.1 EFFECT OF D-RIBOSE-L-CYSTEINE ON TESTICULAR MALONIALDEHYDE (MDA) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There was a significant increase in testicular MDA level in the Alcohol when compared to the control group and Cellgevity group. There is a significant increase in testicular MDA level in the Alcohol + Cellgevity group when compared to the control group and Cellgevity group.

There is a significant decrease in the testicular MDA level in the Alcohol + Cellgevity group when compared to the Alcohol.

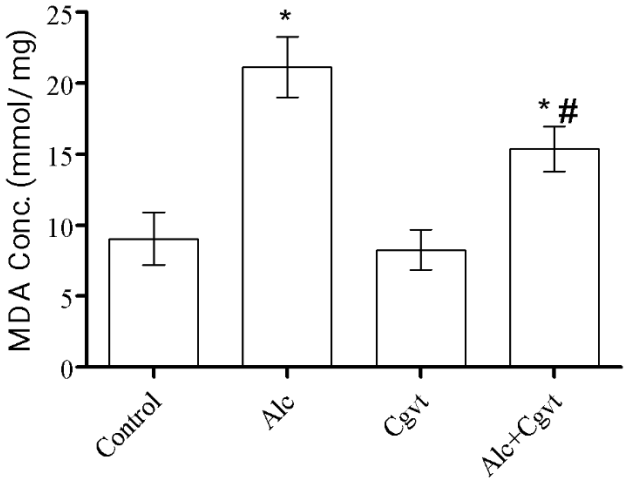


Figure 4.1: shows effect of D-Ribose-L-cysteine on Testicular MDA level in Alcohol induced Sprague-Dawley rats. (Alc=Alcohol, Cgvt=cellgevity and Alc+Cgvt=Alcohol + Cellgevity)

4.2 EFFECT OF D-RIBOSE-L-CYSTEINE ON EPIDIDYMAL MDA LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant increase in epididymal MDA level in the Alcohol group when compared to the control group. There is a significant decrease in epididymal MDA level in the Cellgevity group when compared to the Alcohol group.

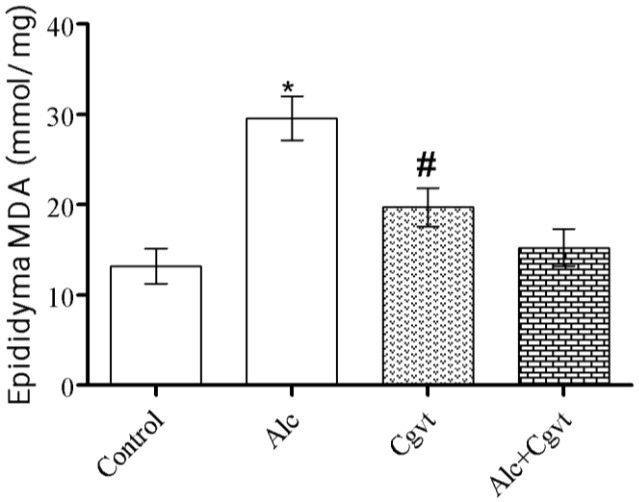


Figure 4.2: Effect of D-Ribose-L-Cysteine on Epididymal MDA level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = Cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.3 EFFECTS OF D-RIBOSE-L-CYSTEINE ON TESTICULAR SUPEROXIDE DISMUTASE (SOD) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in testicular SOD level in the Alcohol group when compared to the control group and Cellgevity group.

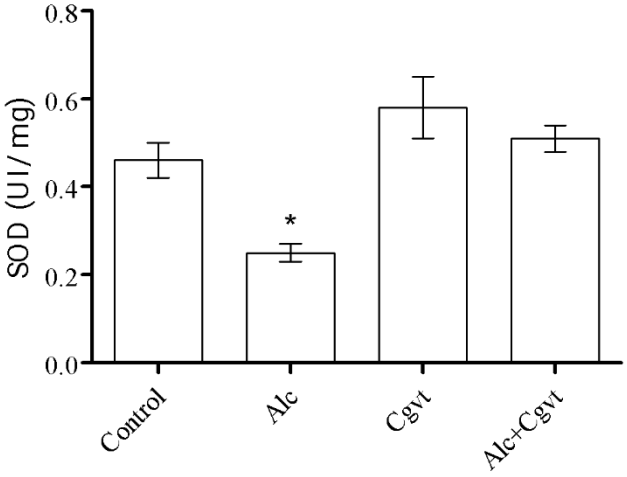


Figure 4.3: Effects of D-Ribose-L-Cysteine on Testicular SOD level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = Cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.4 EFFECT OF D-RIBOSE-L-CYSTEINE ON EPIDIDYMAL SUPEROXIDE DISMUTASE (SOD) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in epididymal SOD level in the Alcohol group when compared to the control group and Cellgevity group. There is a significant increase in epididymal SOD level in Alcohol + Cellgevity group when compared to the Alcohol group.

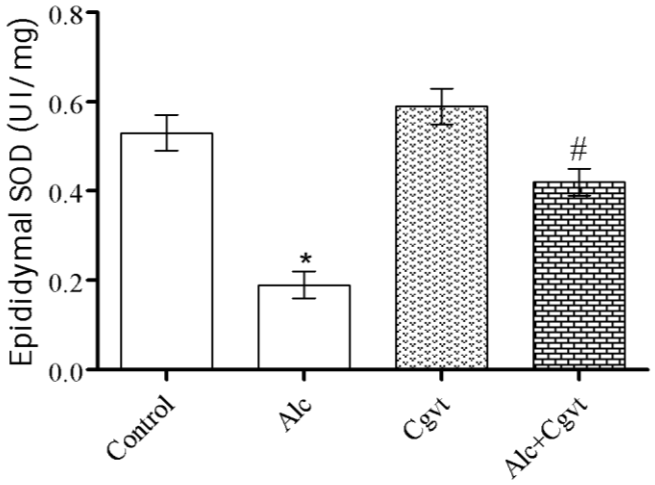


Figure 4.4: Effect of D-Ribose-L-Cysteine on Epididymal Superoxide Dismutase (SOD) level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.5 EFFECTS OF D-RIBOSE-L-CYSTEINE ON TESTICULAR CATALASE (CAT) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in testicular CAT level in the Alcohol group when compared to the control group and the Cellgevity group. There is a significant decrease in testicular CAT level in the Alcohol + Cellgevity group when compared to the control group and the Cellgevity group.

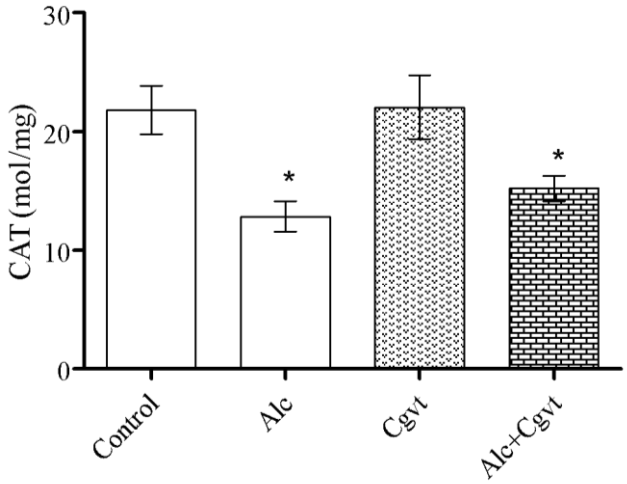


Figure 4.5: Effects of D-Ribose-L-Cysteine on Testicular CAT level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = Cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.6 EFFECT OF D-RIBOSE-L-CYSTEINE ON EPIDIDYMAL CATALASE (CAT) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant increase in epididymal CAT level in Alcohol group when compared to the control group and Cellgevity group. There is a significant increase in epididymal CAT level in Alcohol + Cellgevity group when compared to the control group and Cellgevity group.

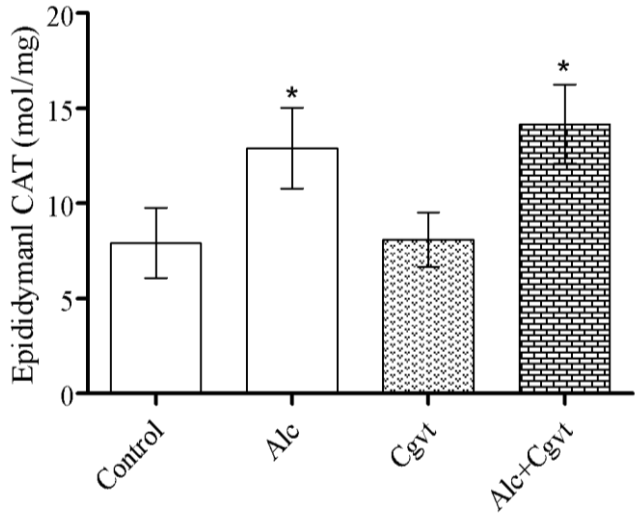


Figure 4.6: Effect of D-Ribose-L-Cysteine on Epididymal Catalase (CAT) level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.7 EFFECT OF D-RIBOSE-L-CYSTEINE ON THE TESTICULAR GLUTATHIONE (GSH) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in testicular glutathione level in Alcohol group when compared to the control group and Cellgevity group. There is a significant decrease in testicular glutathione in Alcohol + Cellgevity group when compared to the control group and Cellgevity group. There is a significant increase in the testicular glutathione level in the Alcohol + Cellgevity group when compared to the Alcohol.

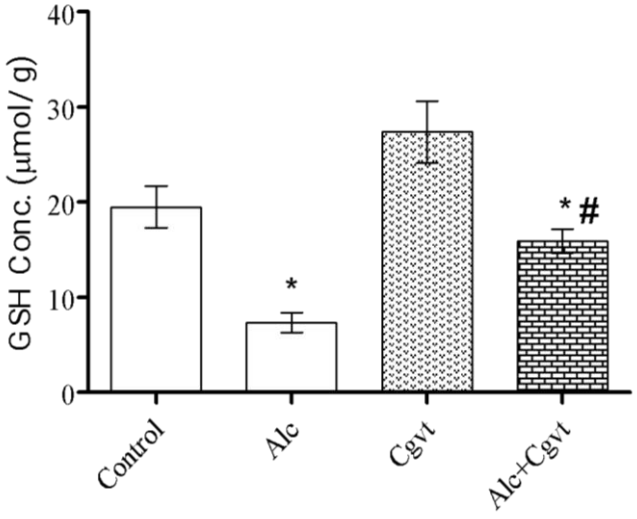


Figure 4.7: Effect of D-Ribose-L-Cysteine on the Testicular Glutathione level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = Cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.8 EFFECT OF D-RIBOSE-L-CYSTEINE ON EPIDIDYMAL GLUTATHIONE (GSH) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in testicular glutathione level in Alcohol group when compared to the control group and Cellgevity group. There is a significant decrease in testicular

glutathione level in Alcohol + Cellgevity group when compared to the control group and Cellgevity group. There is a significant increase in testicular glutathione level in Alcohol + Cellgevity group compared to the Alcohol group.

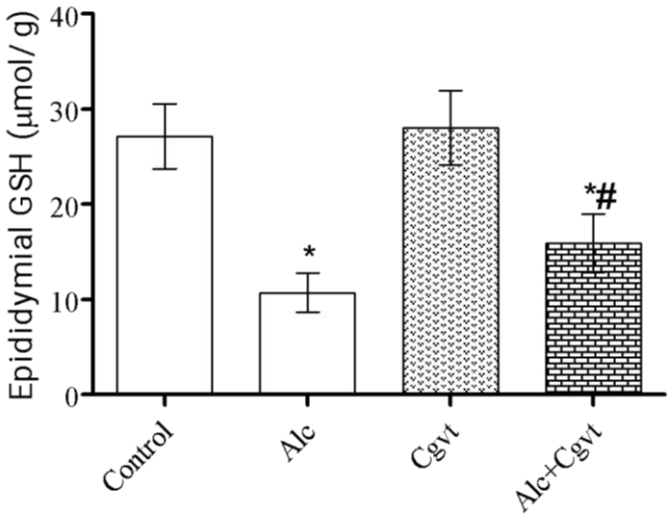


Figure 4.8: Effect of D-Ribose-L-Cysteine on Epididymal Glutathione (GSH) level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.9 EFFECT OF D-RIBOSE-L-CYSTEINE ON SERUM ZINC (Zn) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant increase in serum Zn level in Alcohol group when compared to the Control group and Cellgevity group. There is a significant increase in serum Zn level in Alcohol + Cellgevity group when compared to the Control group and Cellgevity group. However, a slight decrease in serum level in the Alcohol + Cellgevity group when compared with the Alcohol group.

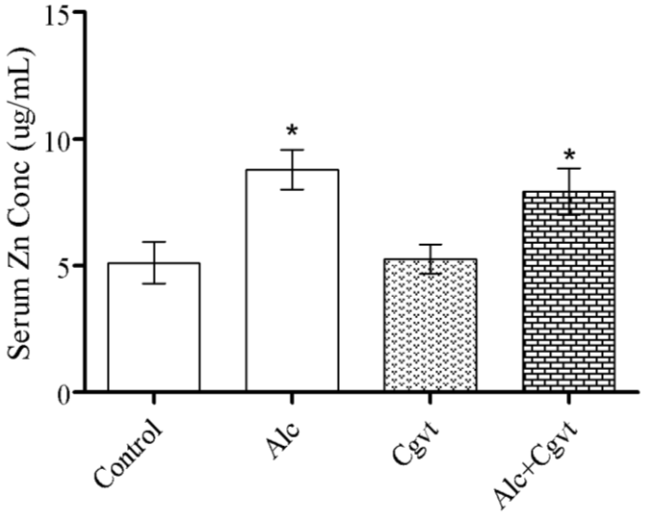


Figure 4.9: Effect of D-Ribose-L-Cysteine on Serum Zinc (Zn)in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.10 EFFECT OF D-RIBOSE-L-CYSTEINE TESTICULAR ZINC LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in testicular Zn level in Alcohol group when compared to the control group and Cellgevity group. There is a significant decrease in testicular Zn level in Alcohol + Cellgevity group when compared to the control group and Cellgevity group. However, a slight increase testicular Zn level in Alcohol + Cellgevity group when compared with the Alcohol group.

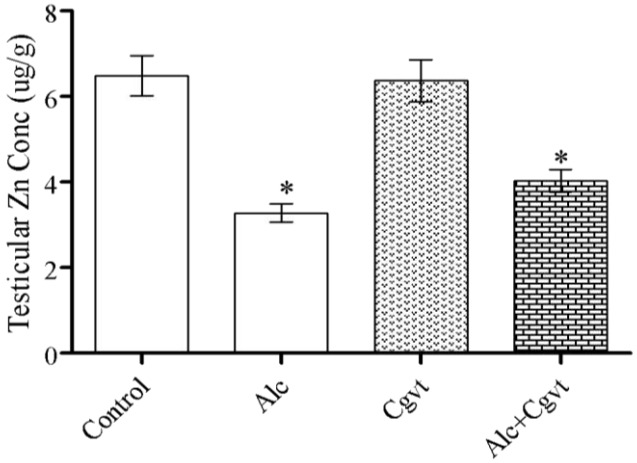


Figure 4.10: Effect of D-Ribose-L-Cysteine on Testicular Zinc (Zn) in Alcohol-induced Sprague-Dawley rats (Alc=Alcohol, Cgvt=cellgevity and Alc+Cgvt=Alcohol + Cellgevity)

4.11 EFFECT OF D-RIBOSE-L-CYSTEINE ON EPIDIDYMAL ZINC (Zn) LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There is a significant decrease in epididymal Zn in the Alcohol group when compared to the control group and Cellgevity group. There is a significant decrease in epididymal Zn in the Alcohol + Cellgevity group when compared to the control group and Cellgevity group. However, a significant increase in epididymal Zn in the Alcohol + Cellgevity group when compared to the Alcohol group.

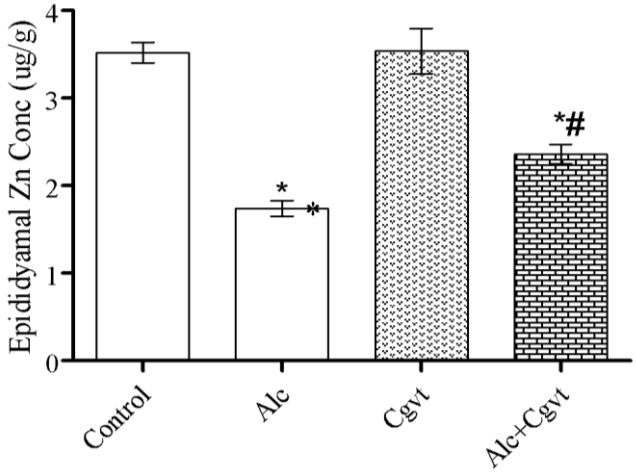


Figure 4.11: Effect of D-Ribose-L-Cysteine on Epididymal Zinc (Zn) level in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.12 EFFECT OF D-RIBOSE-L-CYSTEINE ON SPERM CONCENTRATION LEVEL IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

There are no significant changes on sperm concentration level in any of the group.

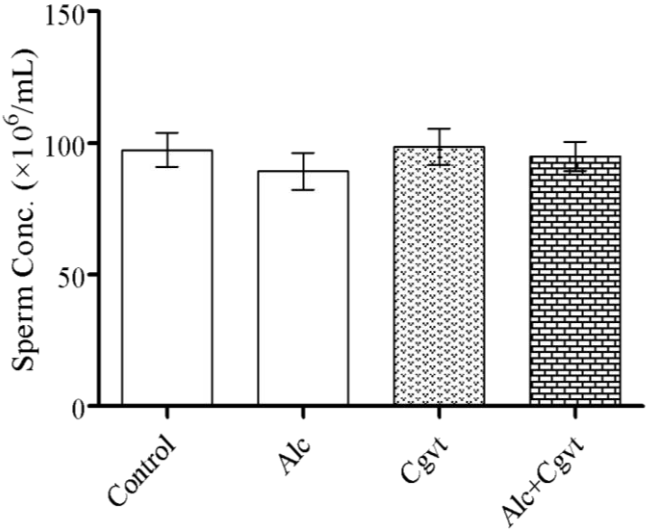
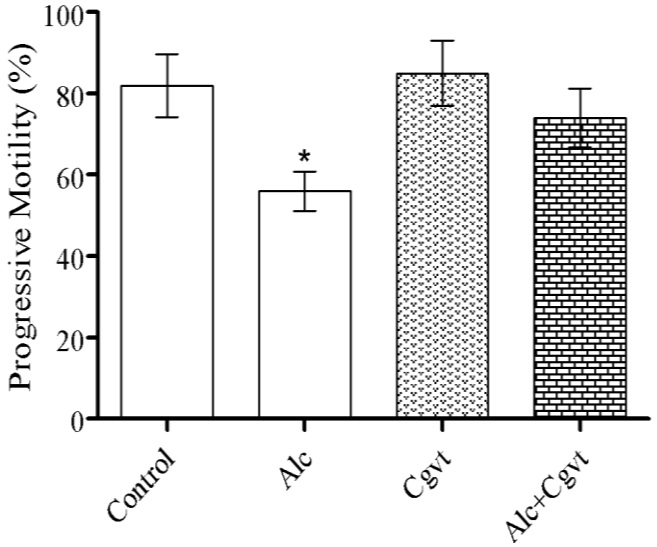


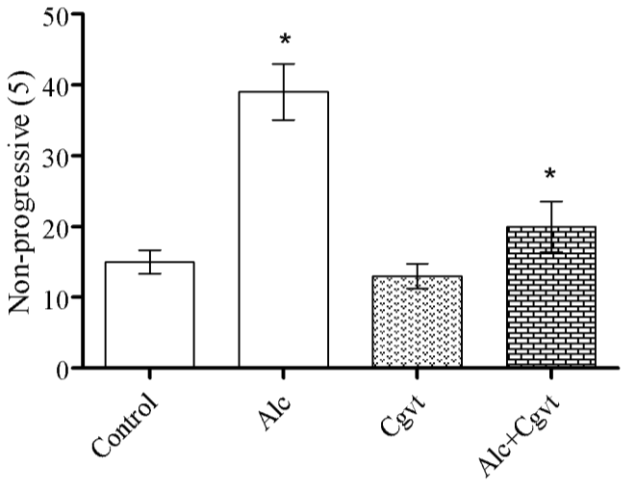
Figure 4.12: Effect of D-Ribose-L-Cysteine on sperm concentration in Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity)

4.13 EFFECT OF D-RIBOSE-L-CYSTEINE SPERM MOTILITY IN ALCOHOL-INDUCED SPRAGUE-DAWLEY RATS

For progressive motility: there is a significant decrease in the sperm motility in the Alcohol group when compared to the control group and Cellgevity group.



For non-progressive motility: there is a significant increase in sperm motility in the Alcohol group when compared to the control group and Cellgevity group. There is a significant increase in sperm motility in Alcohol + Cellgevity group when compared to the Control group and Cellgevity group.



For immotility: there is a significant increase in immotility in Alcohol group when compared to the Control group and Cellgevity group. There is a significant increase in immotility in Alcohol + Cellgevity group when compared to the Control group and Cellgevity group. There is a significant decrease in immotility in the Cellgevity group when compared to all other groups.

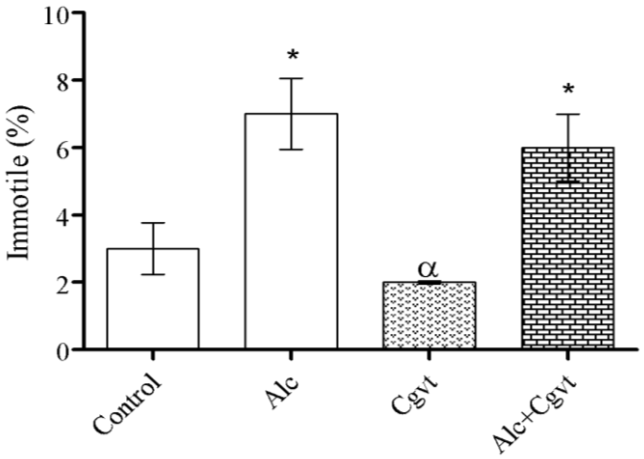


Figure 4.13: Effect of D-Ribose-L-Cysteine Sperm Motility (a. progressive motility b. Non-progressive motility c. Immotility) Alcohol-induced Sprague-Dawley rats (Alc = Alcohol, Cgvt = cellgevity and Alc + Cgvt = Alcohol + Cellgevity).

5. Discussion and Conclusion

The findings of this study indicate that supplementing with D-Ribose-L-Cysteine may be an advantageous prophylactic treatment for male reproductive dysfunction caused by ethanol consumption.

In this current study, the group that received both cellgevity only and ethanol treatment with cellgevity saw a considerable improvement (significant increase) in semen parameters. In particular, when compared to the alcohol group, the cellgevity group and the alcohol + cellgevity group did not exhibit a statistically significant shift in sperm concentration. This suggests that the impact of D-Ribose-L-Cysteine on sperm motility is more pronounced than its influence on sperm concentration. When comparing the cellgevity group and the

alcohol + cellgevity group to the alcohol group, there was a noticeable increase in progressive motility. This beneficial effect of D-Ribose-L-Cysteine is in line with the earlier study conducted by Medubi et al [48]. Furthermore, D-Ribose-L-Cysteine's activity is further enhanced by gradual immotility that only increases in the alcohol group.

This study saw a rise in reactive oxygen species (ROS) due to increased lipid peroxidation, which in turn caused oxidative stress. Furthermore, it has been noted that elevated ROS levels are found in 25–80% of male infertile individuals [49]. The markedly higher MDA levels in the alcohol-only group when compared to the other groups are further demonstrated by the rise in lipid peroxidation. The fundamental cause of oxidative stress is an imbalance between the body's ability to neutralize or detoxify free radicals by neutralizing their detrimental effects through the action of antioxidants. In reversal, superoxide dismutase, catalase, and glutathione were thus significantly higher in the cellgevity group and the alcohol + cellgevity group than in the alcohol group.

The significant decrease in testicular zinc in alcohol group compared to other groups correlates with the report of Baj, et al [50] that alcohol can interfere with the absorption of zinc in the different organs including the digestive tract, leading to reduced uptake of the mineral, increased excretion, internal redistribution, and altered zinc transporters. The increase in serum zinc level in alcohol group compared to other groups confirms zinc reduced cellular absorption and its sequestration in the blood leading to excessive zinc excretion in urine. Absorption of zinc is impaired in acute and chronic alcohol consumers, and ethanol induction to lab rats reduces zinc cellular absorption [51]

In conclusion, these data indicate that ethanol administration impacts sperm motility and morphology, hormones that control sexual behavior (testosterone), trace elements in the testes, and increases oxidative stress. Additionally, the results demonstrate that D-Ribose-L-Cysteine (Cellgevity^{TM}) administration had a protective effect on male reproductive functions by preventing oxidative stress and preventing a significant reduction in testicular zinc even after ethanol induction.

Data Availability (Size 10 Bold)
Not Applicable

Conflict of Interest
Not Applicable

Funding Source
Not Applicable

Authors' Contributions
All co-authors were involved in all stages of this study while preparing the final version. All authors read and approved the final manuscript.

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