Original Article

Protective Effects of Clove Isolates on Testicular Health and Sperm Redox Status in TCA-Treated Rats

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Male reproductive health is increasingly vulnerable to environmental pollutants, with Trichloroacetic acid (TCA) being a common contaminant that adversely affects the male reproductive system. This study investigates the modulating effects of clove isolates, specifically eugenol, on testicular patho-histology and sperm redox status in TCA-treated rats. Known for their antioxidant properties, clove isolates are explored for their potential therapeutic intervention against TCA-induced testicular toxicity. Sixty adult male Wistar rats were divided into ten groups of six rats each: Group 1 received 2 ml/kg body weight of normal saline daily for 30 days via orogastric cannula (Negative Control); Group 2 received 4 mg/kg of EIC via orogastric cannula for 15 days; Group 3 received 10 mg/kg of EIC via orogastric cannula for 15 days; Group 4 received 200 mg/kg of TCA via orogastric cannula for 15 days; Group 5 received 400 mg/kg of TCA via orogastric cannula for 15 days; Group 6 received 200 mg/kg of TCA for the first 15 days, followed by 4 mg/kg of EIC for another 15 days via orogastric cannula; Group 7 received 400 mg/kg of TCA for the first 15 days, followed by 10 mg/kg of EIC for another 15 days via orogastric cannula; Group 8 received 4 mg/kg of EIC for the first 15 days, followed by 200 mg/kg of TCA for another 15 days via orogastric cannula; Group 9 received 10 mg/kg of EIC for the first 15 days, followed by 400 mg/kg of TCA for another 15 days via orogastric cannula; and Group 10 received 4 mg/kg of EIC for 30 days in combination with 200 mg/kg of TCA for 30 days via orogastric cannula. Results indicate that TCA significantly impairs sperm parameters, while eugenol isolates show the potential to mitigate TCA's detrimental effects. Oxidative stress markers reveal a dosedependent response, supporting these findings. Histological observations highlight TCA-induced testicular damage and the protective effects of eugenol isolates. This research provides valuable insights into the protective effects of clove isolates on male reproductive health, suggesting potential natural-based interventions to counter environmental challenges. The study underscores the complex interplay between TCA, clove isolates, and reproductive parameters, offering a foundation for further research and therapeutic interventions to safeguard testicular health.

Keywords: *Spermatozoa, Trichloroacetic acid (TCA), Clove, Eugenol, Testes, Antioxidant, Toxicity*.

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Introduction

Male reproductive health is a crucial aspect of overall well-being, and its vulnerability to environmental pollutants has become a subject of increasing concern in recent years (Aitken *et al*., 1995). Trichloroacetic acid (TCA), a common environmental contaminant resulting from chlorination processes, has been reported to adversely affect the male reproductive system. The detrimental impact of TCA on testicular patho-histology and sperm redox status underscores the urgent need to explore potential therapeutic interventions to mitigate its deleterious effects (Saradha & Mathur, 2006).

The adverse impact of environmental and therapeutic agents on reproductive health is particularly concerning in the context of declining sperm quality in both males and females (EU-OSHA, 2017). Infertility rates are rising, affecting 8-12% of couples worldwide, with male factor infertility contributing to 50% of cases (Agarwal *et al*., 2021). Male infertility can result from congenital malformations, hormonal imbalances, genetic predispositions, behavioral choices, environmental exposures, and lifestyle factors (Karavolos *et al*., 2020).

Environmental factors, especially exposure to air pollution, have been identified as significant contributors to male infertility. These factors impact sperm parameters through the generation of chronic reactive oxygen species (ROS) (Checa *et al*., 2016; Zhang *et al*., 2020). Sperm quality, a crucial determinant of male fertility, declines due to pollution-induced impairments in steroidogenesis, spermatogenesis, Sertoli cells, and overall sperm function (Nateghian & Aliabadi, 2020; Selvaraju *et al*., 2021). While spermatozoa naturally produce ROS as part of early signaling for sperm capacitation, excessive ROS levels from environmental toxicants disrupt the prooxidant/antioxidant balance, adversely affecting testicular function (Aitken *et al*., 1995; Saradha & Mathur, 2006).

Clove (Syzygium aromaticum) has been recognized for its diverse pharmacological properties, including antioxidant, anti-inflammatory, and antimicrobial activities (Grinsburg, 2003). The rich phytochemical composition of clove, particularly its isolate eugenol, has been the focus of extensive research due to its potential health-promoting effects. Recent studies suggest that clove isolates may protect against oxidative stress-induced damage in various tissues, making them promising candidates for preserving male reproductive health (Mapanga *et al*., 2009; Madlala *et al*., 2012; Liu *et al*., 2015).

This research investigated the modulating effects of clove isolates, specifically eugenol, on the testicular patho-histology and sperm redox status in TCA-treated rats. The rationale behind this study is grounded in growing evidence highlighting the therapeutic potential of natural compounds in ameliorating the adverse effects of environmental pollutants on reproductive health.

Several studies have implicated TCA exposure in the disruption of spermatogenesis, induction of oxidative stress, and alteration of testicular architecture (Mishra & Singh, 2013; Choi *et al*., 2014; Moghimian, 2017). The precise mechanisms underlying these effects remain to be fully elucidated,

providing an opportunity for targeted interventions. Clove isolates, with their well-established antioxidant properties, may offer a promising avenue for therapeutic intervention against TCAinduced reproductive toxicity.

This study aims to contribute to the existing body of knowledge by providing insights into the potential protective effects of clove isolates on testicular function and sperm redox status in the context of TCA exposure. The findings may have significant implications for the development of natural-based interventions to safeguard male reproductive health against environmental challenges.

Materials and Methods

Experimental Animals

A total of Sixty (60) adult male Wistar rats weighing an average of 110g were procured from the Animal House of the College of Health Science, Benue State University Makurdi and were allowed to acclimatize for fourteen (14) days in mesh net-covered plastic cages in ten (10) groups of six (6) and given *ad libitum* access to grower Vital feed pellet and water before the commencement of the experiment. The weights of rats were measured at acquisition, during acclimatization, before and after administration of the extract, and at the end of the experiment using an electronic weighing balance.

Animal Cages

A total of ten (10) plastic cages measuring 30cm×20cm in size were obtained, in which the experimental animals were housed, acclimatized, and fed throughout the experiment.

Trichloroacetic Acid (TCA)

Trichloroacetic acid and sodium hydroxide pellets, that were used to neutralize TCA solution (K_a=0.3) to required pH 7.0–7.5, were purchased from a chemical shop in Abuja. The purity of TCA and sodium hydroxide was ensured to be >99.0%. Trichloroacetic acid is stable in a neutral solution and is classified as non-biodegradable.

Animal Feeds

The animal feed (UAC Vital feed Grower made in Nigeria) was purchased from a feed store in Wurukum area of Makurdi and stored at optimum temperature in the animal house.

Methodology of Eugenol Extraction from Clove Oil

Chemicals

Clove bud species (*Syzygium aromaticum*) were utilized. Chemicals, particularly solvents, were utilized in the process of extracting essential oils, in the creation of potential environmentally acceptable packaging, or during characterization. Merck Chemicals provided the technical grade 96% n-hexane, glacial p.a. 100% acetic acid, p.a. 99.9% ethanol, and p.a. 99.0% acetone. Industrial grade chitosan from CV. ChiMultiguna, with particle sizes ranging from 30 to 80 mesh, was used to make pulp. To increase the mechanical qualities, the used paper was incorporated. HVS 80 gr. sheets were the type of paper utilized in the experiment.

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Instrumentations

To characterize and assess the properties of the materials, various devices were utilized, including Thermogravimetric Analysis (TGA), Fourier Transform Infrared (FTIR), Universal Testing Machine (UTM), and Gas Chromatography-Mass Spectrometer (GC-MS). The essential oil content was identified using Thermo Trace 1310 GC with Mass Spectrometer, Thermo ISQ Single Quad Detector, and FTIR Spectrometer System Nicolet iS 5 in Attenuated Total Reflectance (ATR) mode. Mechanical characteristics of the potential green paper were evaluated through Material Strength Testing using Zwick Roell Z100. The thermal deterioration of the eco-friendly paper was examined using the Discovery-650 SDT (Simultaneous DSC-TGA).

Extraction of Clove Oil

To separate clove oil, steam hydro distillation is chosen as the technique. 18kg of dried clove buds were utilized. The steam distillation took place throughout 3, 4, 5, and 6 hours. When the first drop of distillate was released, the clock began to run. The recovered distillate was then extracted once again using a separatory funnel and n-hexane as the solvent. N-hexane was evaporated to produce clove oil.

Characterization of Clove Oil

FTIR and GC-MS spectrum analysis was used to analyze the content and properties of clove oil, and the results were then compared to commercial products of 100% pure clove oil. ATR-FTIR measurements of a few drops of clove oil were performed to compare the compounds' functional group similarities. GC-MS has been utilized to assess the clove oil contents based on mass-tocharge (m/z) measurements in addition to ATR findings. Trace GOLDTM TG-1MS column (length 30 m; ID 0.25 mm; film thickness 0.25 m) was used to separate clove oil. Using a split ratio of 1/50, a 1L sample that had been diluted by 1% in methanol was put onto the column.

The instrument approach has been enhanced using gradient elution to provide effective compound separation. The system was initially brought to equilibrium at 50^{o}C. The temperature was then progressively increased to 100°C by adding 10°C per minute, held for 1 minute, then increased to 140°C by adding 5°C per minute, holding for 1 minute, then increased to 160°C by adding 2°C per minute, holding for 1 minute, and lastly increased to 245°C by adding 5°C per minute, holding for 1 minute. By using the electrospray ionization mode (EI), the mass-to-charge (m/z) of the chemicals found in clove oil was discovered. The ion source temperature was kept at 250°C, while injector and detector temperatures were set to 280°C.

Helium gas, used as the mobile phase and flowing at a rate of 1 mL/min, was used to elute and segregate the sample down the column. By comparing the m/z of clove oil to the mass spectra in their collection (NIST MS), the chemical components are identified.

In many hours, clove oil was extracted using the steam hydro distillation process, and then the distillate was removed using a separatory funnel and n-hexane. In 6 hours, the observed maximum % yield of extracting clove oil was reached; the yield was 7.04%. Using FTIR, the FTIR spectra

of the 100% pure clove oil (*Syzygium aromaticum*) that was commercially available were compared to the spectra of the clove oil that was obtained via steam hydro distillation for 6 hours. The extracted clove bud sample and the commercial essential oil sample show FTIR spectra that are quite close to one another, with a similarity of 98.88%.

Experimental Design

The sixty (60) adult male Wistar rats were divided into ten (10) groups of six (6) rats each, and administered the research substances as follows:

Group 1 - Negative Control (Placebo): 2 ml/kg body weight of normal saline daily for 30 days through an orogastric cannula.

Group 2 - EIC Low Dose: 4 mg/kg of EIC via orogastric canula for 15 days.

Group 3 - EIC Standard Dose: 10 mg/kg of EIC for 15 days via orogastric canula.

Group 4 - TCA Low Dose: 200 mg/kg of TCA for 15 days through an orogastric cannula.

Group 5 - TCA High Dose: 400 mg/kg of TCA for 15 days through an orogastric cannula.

Group 6 - TCA Low Dose + EIC Low Dose: 200 mg/kg TCA for the first 15 days + 4 mg/kg of EIC for another 15 days through an orogastric cannula.

Group 7 - TCA High Dose + EIC Standard Dose: 400 mg/kg TCA for the first 15 days + 10 mg/kg of EIC further 15 days through an orogastric cannula.

Group 8 - EIC Low Dose + TCA Low Dose: 4 mg/kg of EIC for the first 15 days + 200 mg/kg of TCA for another 15 days via orogastric cannula.

Group 9 - EIC Standard Dose + TCA High Dose: 10 mg/kg of EIC for first 15 days + 400 mg/kg of TCA for another 15 days through an orogastric cannula.

Group 10 - EIC Standard Dose + TCA Low Dose (Extended): 4 mg/kg of EIC for 30 days in combination with 200 mg/kg of TCA for 30 days through an orogastric cannula.

Animal Sacrifice

Upon sacrifice, the rats were weighed before decapitation. Following sacrifice, blood samples were promptly collected from the heart of each rat. A midline abdominal incision was made to expose the reproductive organs. The testes and epididymis were excised, and the weight of each animal's testes was assessed using an electronic analytical and precision balance.

Testis volume was determined using the water displacement method. Both tests of each rat were measured, and the average value for each parameter was considered as one observation. One of the testes from each animal was preserved in Bouin’s fluid for subsequent histological examination. Serum and the remaining testis of each animal were stored at –25^{o}C for biochemical assays.

Sperm Analysis

Epididymal Spermatozoa Concentrations

The epididymis' spermatozoa were counted using an altered version of Yokoi and Mayi's 2004 technique. The epididymis was chopped with anatomic scissors in 5 milliliters of physiologic saline, set in a rocker for 10 minutes, and then incubated for two minutes at room temperature.

Following incubation, a solution containing 1 mL of 35% formalin and 5 g of sodium bicarbonate was used to dilute the supernatant fluid 1:100. Utilising the recently upgraded Neubeur's counting chamber (hemocytometer), the total amount of sperm was ascertained. Five minutes were given to each counting chamber of the hemocytometer after 10 µL of the diluted sperm solution was added. After that, a binocular light microscope was used to illuminate this chamber with an adjustable light source. Spermatozoa were counted in five 16-cell squares within the governed portion of the chamber. The quantity that was computed, multiplied by five, and represented as [X] x 10^6 /ml—where [X] is the number of spermatozoa in a 16-cell square—was the sperm concentration.

Normal and Abnormal Spermatozoa Morphology

Using x40 magnification light microscope. Sperm were extracted from the first dilution and diluted 1:20 with neutral buffered formalin (10%; Sigma-Aldrich, Oakville, ON, Canada) for motility testing. For every sample, five hundred sperm were analyzed for any anomalies in their morphology. All spermatozoa were classified in wet preparations using phase-contrast optics. A spermatozoon was deemed morphologically aberrant in this study if it possessed one or more of the following characteristics: a detached head, round head, or rudimentary tail. This was expressed as a proportion of sperm that had normal morphology.

Biochemical Analysis

Assay of Superoxide dismutase (SOD) activity

Rukmini *et al.* (2004) reported that superoxide dismutase activity was tested using the Winterbourn *et al.* (1975) technique. The assay's basic idea was based on SOD's capacity to prevent nitro-blue tetrazolium (NBT) from being reduced. In summary, the reaction mixture included 0.1 ml of enzyme samples, 0.05 ml of 0.12 mM riboflavin, 0.1 ml of 1.5 mM NBT, 0.05 ml of 0.01M methionine, and 2.7 ml of 0.067M phosphate buffer at pH 7.8. To guarantee even lighting of the tubes, they were placed in a box with a 15W fluorescent bulb and covered with airfoil for ten minutes. Control without the enzyme source was included. The absorbance was measured at 560nm. The quantity of enzyme needed to prevent the decrease of NBT by 50% under the given circumstances was defined as one unit of SOD. Units of the enzyme's activity were represented as mg of protein.

Estimation of Lipid Peroxidation (Malondialdehyde (MDA)

By using Buege and Aust's thiobarbituric acid reactive substances (TBARS) technique, colorimetric measurements of the tissue's lipid peroxidation were made (1978). Lipid peroxidation produces malondialdehyde (MDA), which is a major component of TBARS. In summary, 2 ml of the 1:1:1 ratio TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl, and 15% TCA) was added to 0.1 ml of tissue in Tris-HCl buffer, pH 7.5. The tissue was then put in a water bath for 15 minutes and allowed to cool. At 535 nm, the absorbance of the clear supernatant was measured in comparison to the reference blank. Malondialdehyde's molar absorptivity of 1.56 x10^5 M^{-1} cm^{-1}, which is represented as nmol/mg protein, was used to compute the concentration.

Assay of Catalase (CAT) activity

The Aebi (1983) technique was used to test the catalase activity. 0.1 ml of tissue was pipetted into a cuvette that held 1.9 ml of pH 7.0, 50 mM phosphate buffer. The addition of 1.0 ml of recently made 30% (v/v) hydrogen peroxide (H_2O_2) initiated the reaction. Using spectrophotometry, the rate of H_2O_2 breakdown was determined by monitoring changes in absorbance at 240 nm. Units of the enzyme's activity were represented as mg of protein.

Haematoxylin and Eosin (H&E) Tissue Processing

The fixed specimens were processed overnight for dehydration, clearing, and impregnation using an automatic tissue processor (Sakura, Japan). The specimens were embedded in paraffin blocks using an embedding station (Sakura, Japan) and serial sections of 5um thickness were cut using a microtome (ModelRM2245, Leica Biosystems, Wetzlar, Germany). We used an autostainer (Model 5020, Leica Biosystems, Wetzlar, Germany) for Hematoxylin & Eosin staining of the sections. The mounted specimens were observed and were scored under light microscopy at x40.

Statistical Analysis

For each number, the mean and standard error of the mean (S.E.M.) were determined. Duncan's multiple range tests were used in conjunction with a one-way analysis of variance (ANOVA) to compare the treatment and control groups. At p~0.05, differences were deemed statistically significant.

Ethical Clearance

Ethical approval was sought and obtained from the Human Research and Ethical Committee (HREC), College of Health Science, Benue State University, Makurdi with clearance number 08038619526. All experimental procedures carried out were following the guidelines on animal experiments as prescribed by the Ethics Committee.

Results

Assessment of Sperm Quality: Sperm Count and Morphology

The rat models subjected to exclusive TCA treatment exhibited noteworthy changes in various sperm parameters. There was a substantial and statistically significant ($p \leq 0.05$) decrease in sperm count and normal sperm morphology, accompanied by a significant ($p \leq 0.05$) increase in abnormal sperm morphology when contrasted with the negative control group (Group 1). These alterations in sperm characteristics highlight the detrimental effects of TCA treatment on reproductive parameters.

Conversely, the groups treated with EIC displayed a striking contrast in these parameters. There was a significant increase in sperm count and a decrease in abnormal sperm morphology, signifying a potentially beneficial impact when compared to the group treated solely with TCA. This suggests the potential of EIC to counteract some of the negative effects of TCA on sperm quality.

It's worth noting that Group 6 exhibited a non-significant ($p > 0.05$) increase in normal sperm morphology when compared to the positive control group. While this increase wasn't statistically

significant, it does hint at a potential positive influence of the treatment in this specific context. In comparing the corresponding dose groups, only Group 7 and Group 9 demonstrated statistically significant ($p \leq 0.05$) changes. Group 7 showed a decrease in normal sperm morphology, while Group 9 exhibited an increase in normal sperm morphology along with changes in abnormal sperm morphology. These specific responses highlight the dose-dependent nature of the treatment effects on sperm morphology.

Additionally, the classification of sperm morphology revealed no statistically significant differences between the negative and positive control groups and the experimental groups. This implies that the morphological aspects of sperm were not significantly affected by the experimental treatments, as observed in the data. Overall, the findings shed light on the complex interplay between TCA, EIC, and sperm morphology, with both detrimental and potentially beneficial effects on various parameters.

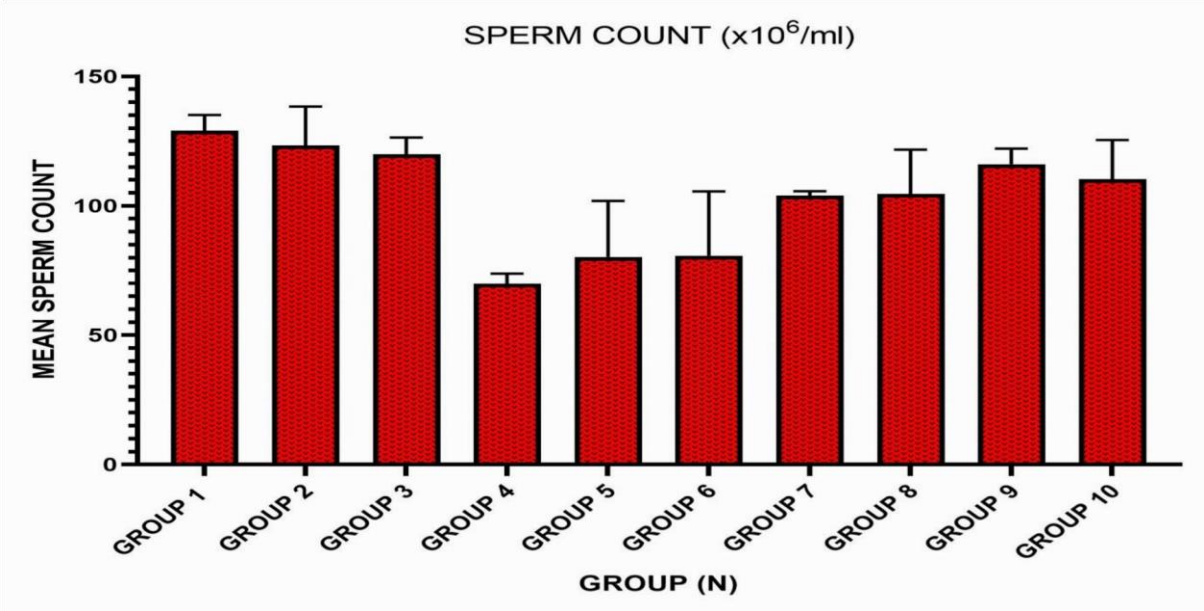

Figure 1: Simple Bar Chart Showing the Mean Sperm Count across groups with significant decreases or increases at $P \leq 0.05$ when compared to group 1 (negative control), groups 4&5 (positive controls), groups 2, 4,6 and 8 (low dose group). N = 4.

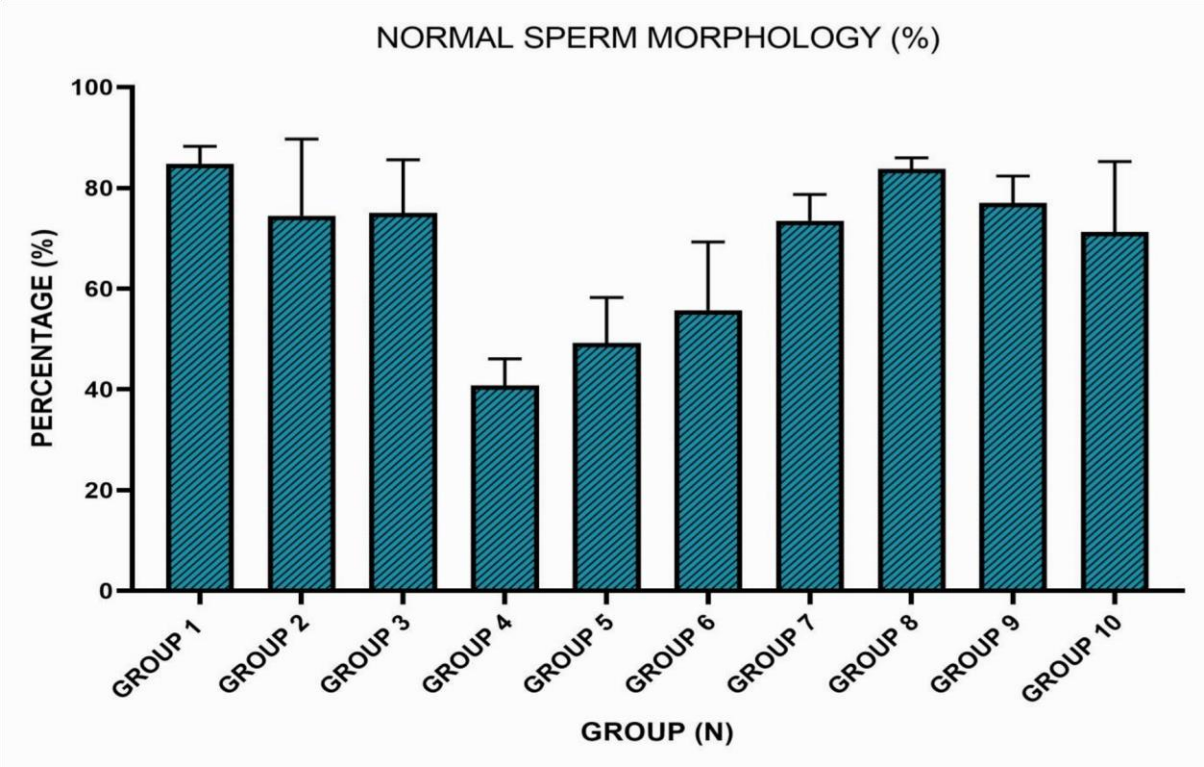

Figure 2: Simple Bar Chart Showing the Mean Normal Sperm Morphology across groups with significant decreases or increases at P≤0.05 when compared to group 1 (negative control), groups 4&5 (positive controls), groups 2, 4,6, and 8 (low dose group). N = 4.

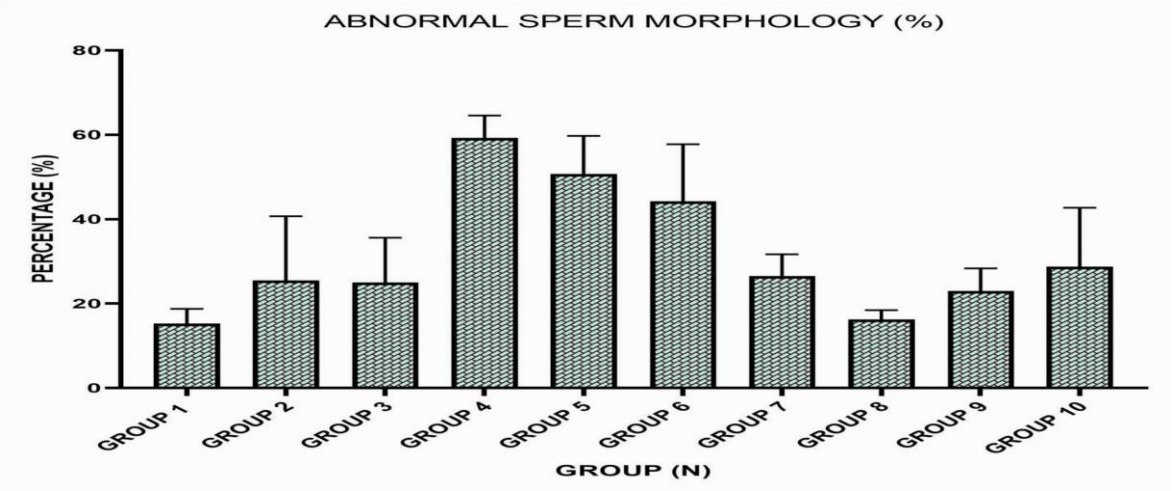

Figure 3: Simple Bar Chart Showing the Mean Abnormal Sperm Morphology across groups with significant decreases or increases at P≤0.05 when compared to group 1 (negative control), groups 4&5 (positive controls), groups 2, 4,6 and 8 (low dose group). N = 4.

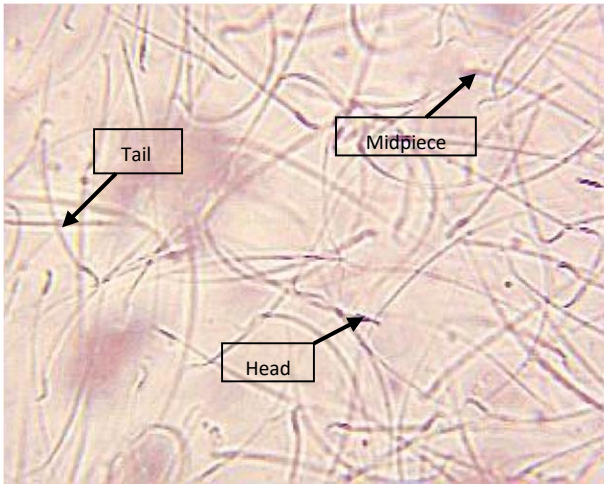

Plate 1: Photomicrograph of sperm from group 1 showing the Head, Tail, and 2 showing the Head, Tail, and Midpiece**. Stain:** Papanicolaou**;** Papanicolaou; **Magnification**: x100.

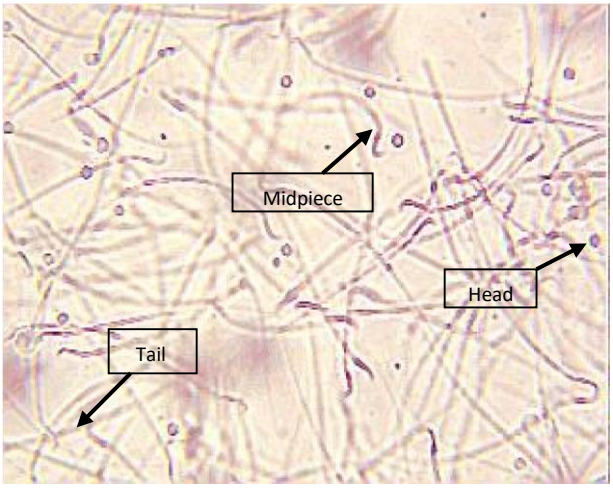

Plate 2: Photomicrograph of sperm from group 1 showing the Head, Tail, and Midpiece**. Stain:** Midpiece**.**

Magnification : x 100

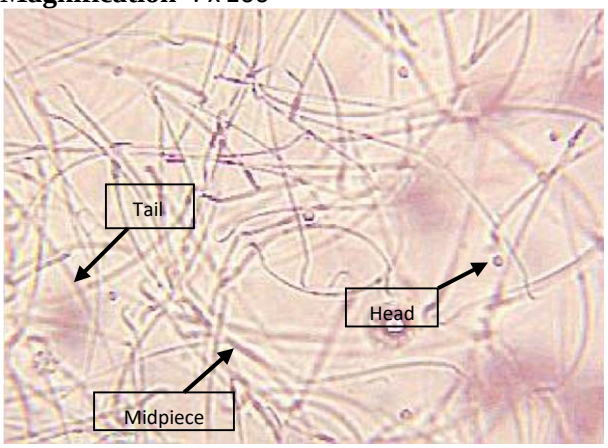

Plate 3: Photomicrograph of sperm from group 3 showing the Head, Tail, and 4 showing the Head, Tail, and Midpiece**. Stain:** Midpiece**.** **Stain:** Papanicolaou**;** Papanicolaou; **Magnification**: x100.

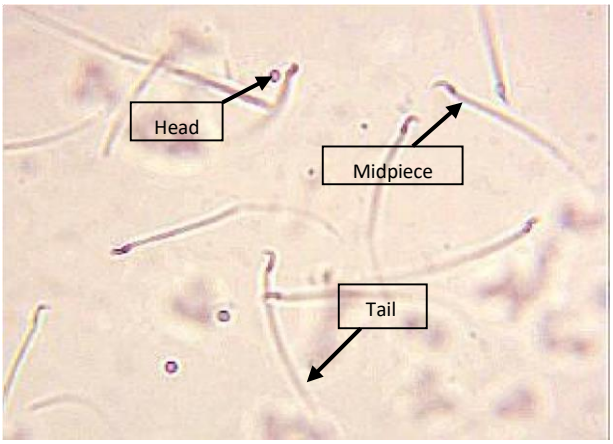

Plate 4: Photomicrograph of sperm from group 3 showing the Head, Tail, and Midpiece**. Stain:** Midpiece**.** **Stain:** Papanicolaou**;** Papanicolaou; **Magnification**: x100.

Magnification: x100.

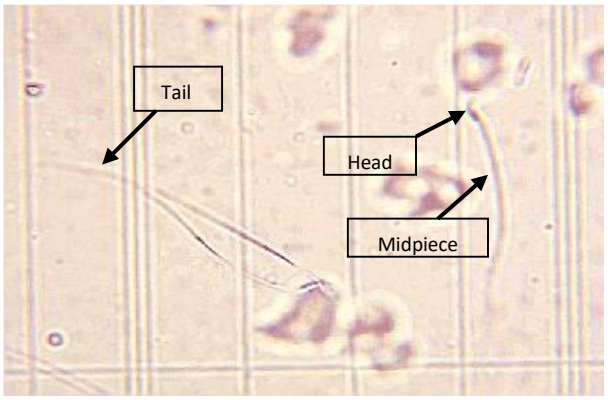

Plate 5: Photomicrograph of sperm from group 5 showing the Head, Tail, and Midpiece**. Stain:** Papanicolaou; **Magnification**: x100.

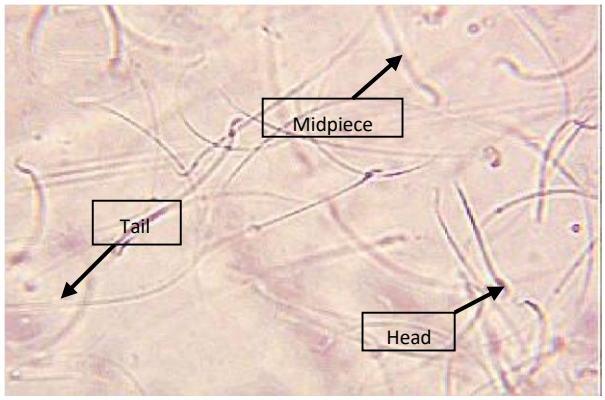

Plate 6: Photomicrograph of sperm from group 6 showing the Head, Tail, and Midpiece**. Stain:** Papanicolaou; **Magnification**: x100.

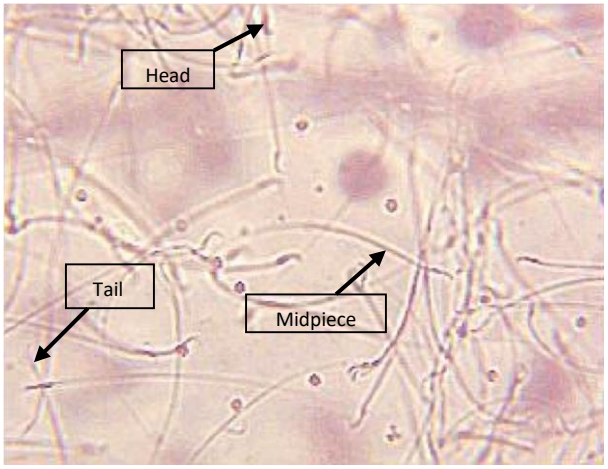

Plate 7: Photomicrograph of sperm from group 7 showing the Head, Tail, and Midpiece**. Stain:** Papanicolaou; **Magnification**: x100.

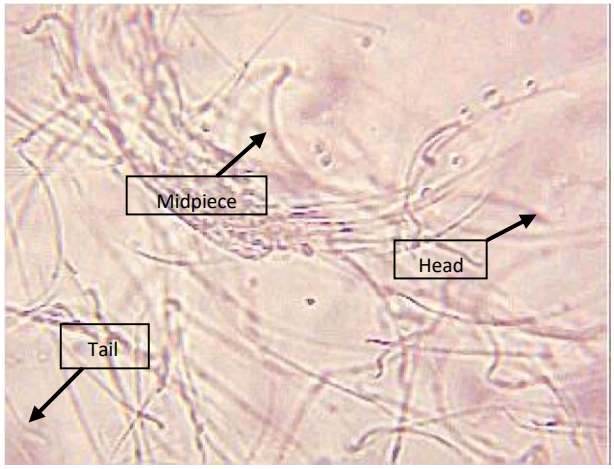

Plate 8: Photomicrograph of sperm from group 8 showing the Head, Tail, and Midpiece**. Stain:** Papanicolaou; **Magnification**: x100.

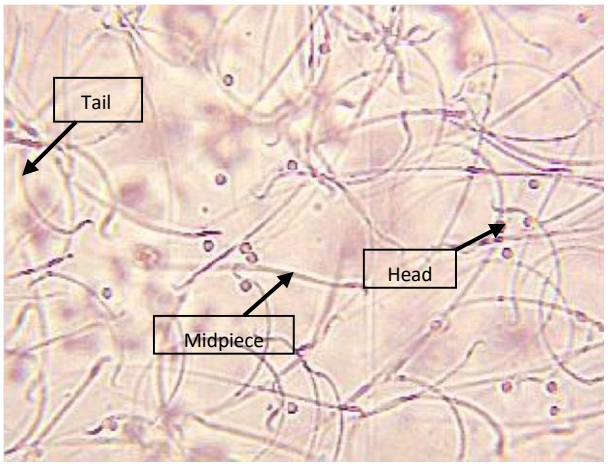

Plate 9: Photomicrograph of sperm from group 9 showing the Head, Tail, and Midpiece Midpiece**.Stain:**Papanicolaou; **Magnification**: x100.

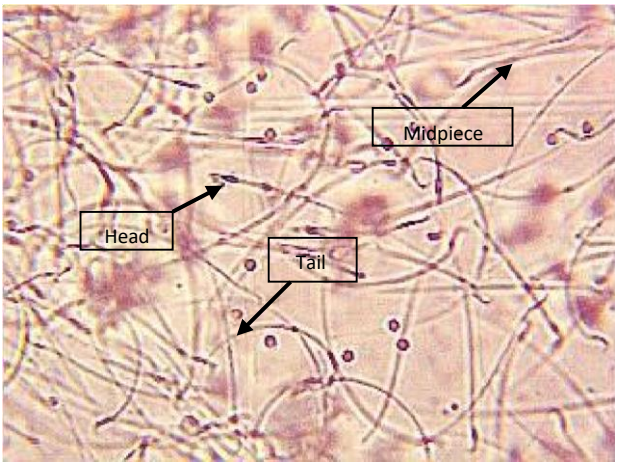

Plate 10: Photomicrograph of sperm from group 10 showing the Head, Tail, and Midpiece **Stain:** Papanicolaou; **Magnification**: x100.

Biochemical Analysis

Oxidative Stress Markers: SOD, CAT and MDA

As depicted in figures 4 - 6, the positive control group exhibited a noteworthy reduction in SOD and CAT levels, coupled with a substantial increase in MDA levels when compared to the negative control group. Moreover, the results revealed a significant elevation in SOD and CAT levels and a marked decrease in MDA levels among the treatment groups (groups 6-8) in comparison to the positive control group. Notably, when comparing the higher doses of TCA (group 5) and EIC, it is evident that the higher dose of EIC, in conjunction with TCA, induced a more pronounced reduction in SOD levels and MDA levels, respectively, compared to their corresponding lower doses.

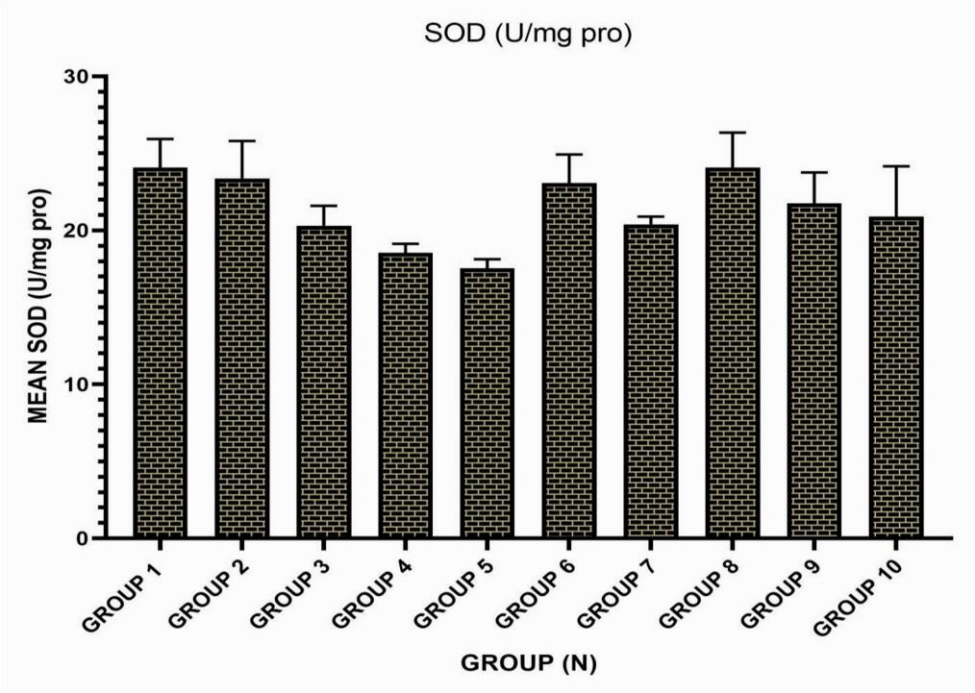

Figure 4: Simple Bar Chart Showing the Mean Superoxide dismutase across groups treated with normal saline for Group 1, EIC, TCA, and EIC, TCA combinations respectively for the rest of the groups.

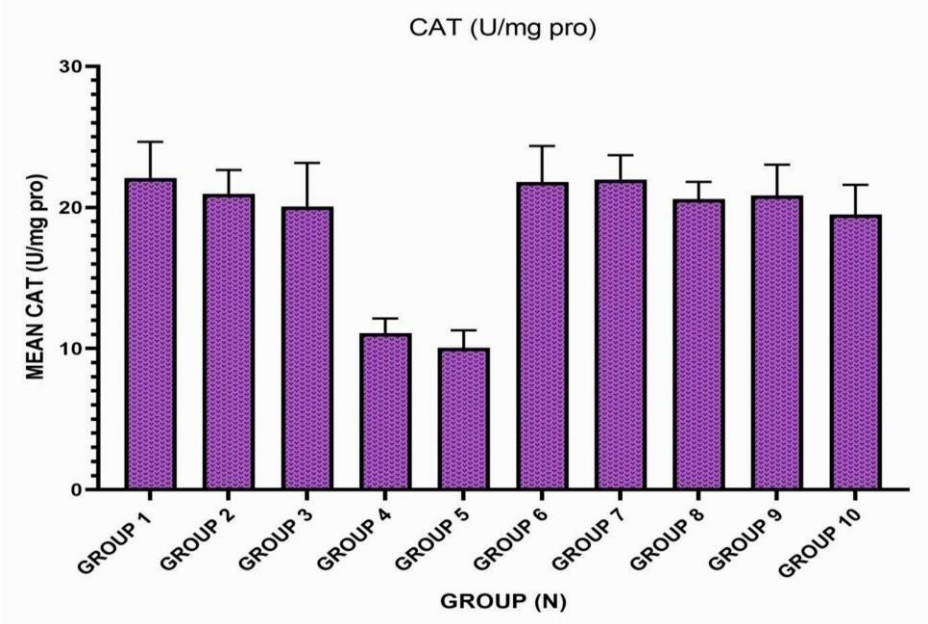

Figure 5: Simple Bar Chart Showing the Mean Catalase across groups treated with normal saline for Group 1, EIC, TCA, and EIC, TCA combinations respectively for the rest of the group.

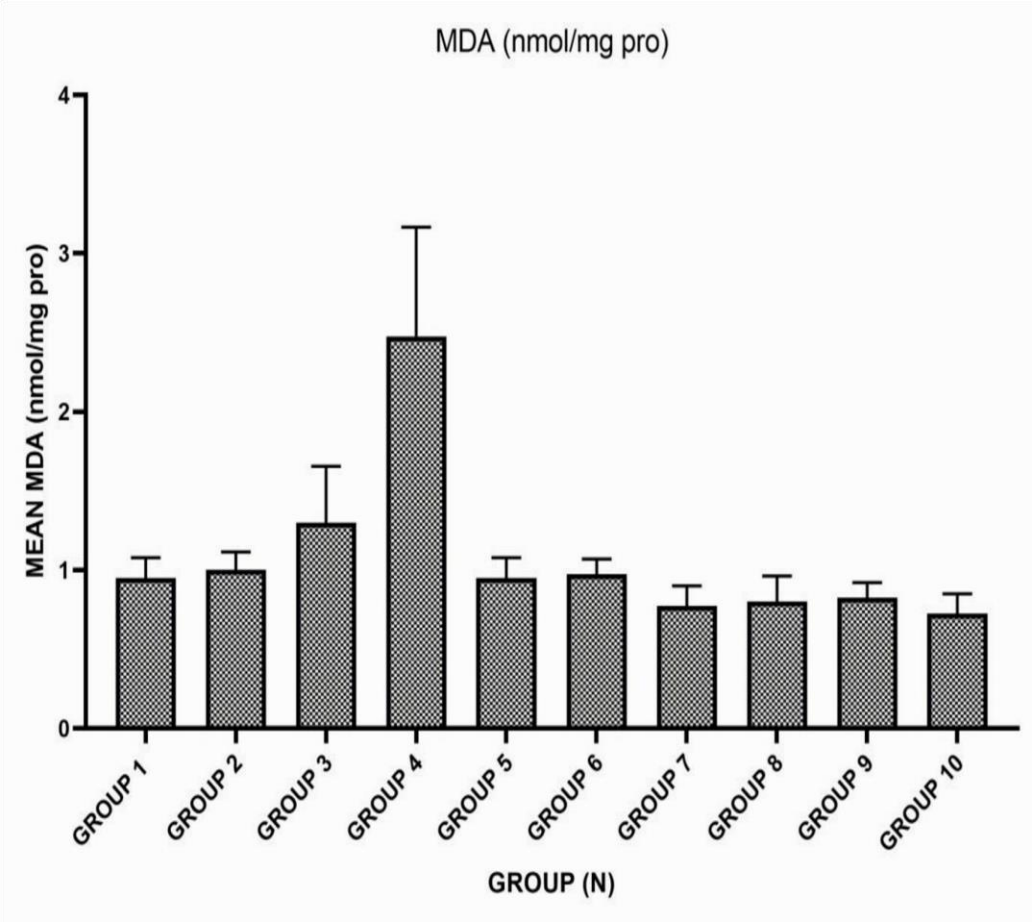

Figure 6: Simple Bar Chart Showing the Mean Malondialdehyde across groups treated with normal saline for Group 1, EIC, TCA, and EIC, TCA combinations respectively for the rest of the group.

Histological Observations

Histological observations of the testicular tissue in groups 1 - 3 revealed normal histological characteristics, with an abundance of spermatozoa extending towards the lumen. The Leydig cells remained intact, and spermatid retention was evident within the seminiferous tubules (Plate 11 - 13).

In contrast, groups 4 and 5 exhibited abnormal seminiferous tubule morphology, marked by spermatid retention, tubular atrophy, and widespread disorganization of germ cells. The testicular architecture showed signs of degeneration, with the absence of interstitial space and areas of necrosis. Additionally, several maturing spermatogenic cells were observed within the seminiferous tubules. Some cells exhibited nuclear membrane rupture, accompanied by nucleus fragmentation (karyorrhexis) (Plate 14 - 15).

Conversely, groups 6 - 10 displayed distinct features, characterized by spermatogonia cells with deeply stained nuclei and damaged sperm cells. The majority of seminiferous tubules appeared shrunken with a wavy outline. Thickening and hyalinization of basement membranes were evident, and the lumens of the seminiferous tubules were primarily occupied by fragments of disintegrated apart and preclude direct comparison to the histological features of groups 1-3.

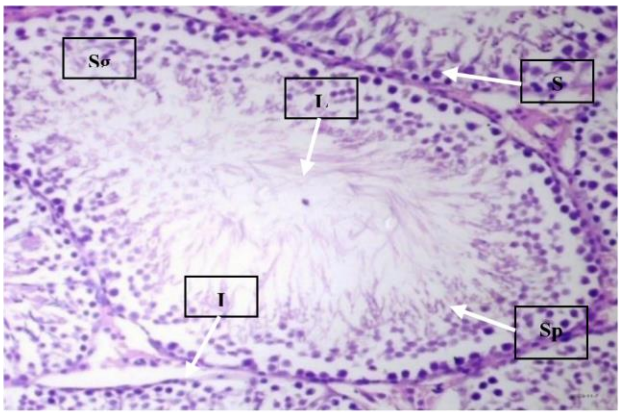

Plate 11: Photomicrograph of testes from group 1 **showing** the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

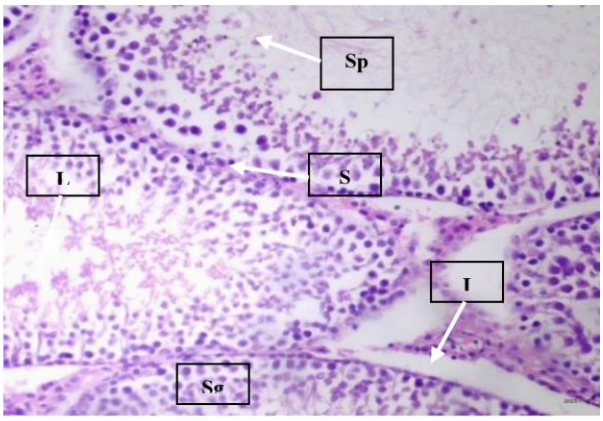

Plate 12: Photomicrograph of testes from group 2 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

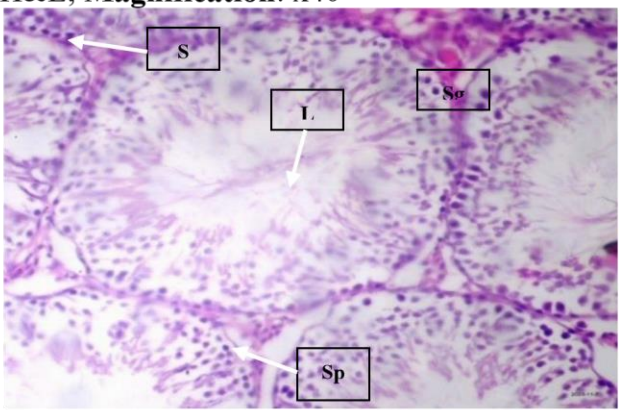

Plate 13: Photomicrograph of testes from group 3 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) **Stain:** H&E; **Magnification**: x40

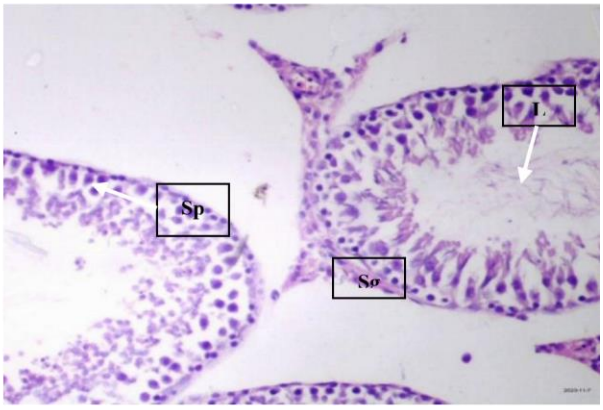

Plate 14: Photomicrograph of testes from group 4 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

cells. Although there were some similarities to groups 4 and 5, these observations set groups 6 -10

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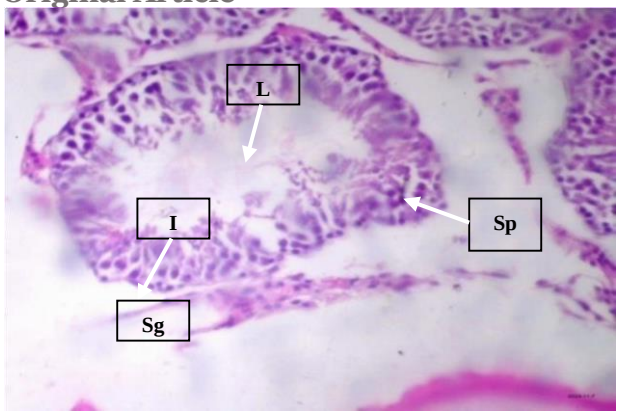

Plate 15: Photomicrograph of testes from group 5 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**. Stain:** H&E; **Magnification**: x40

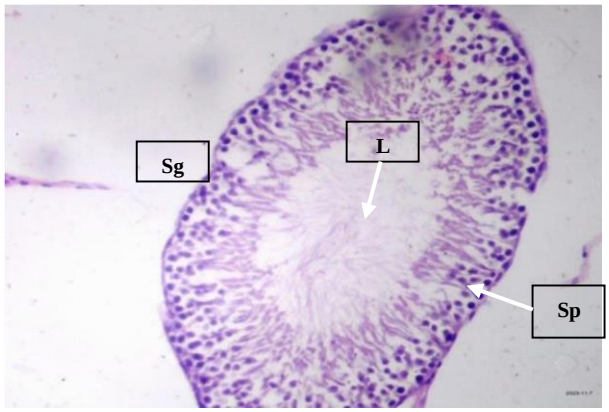

Plate 16: Photomicrograph of testes from group 6 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**.**

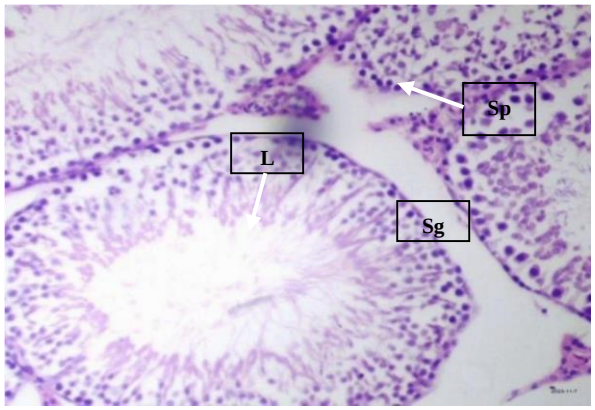

Plate 17: Photomicrograph of testes from H&E; **Magnification**: x40

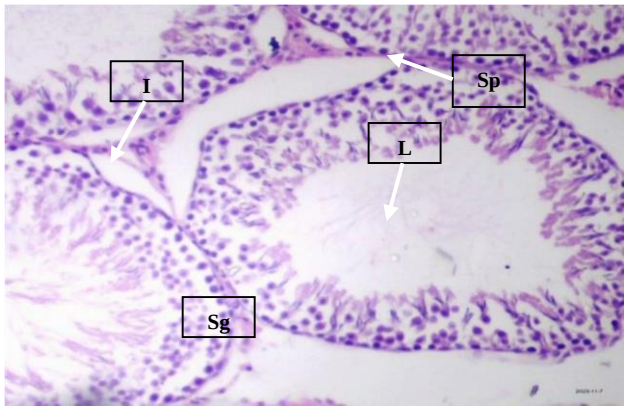

Plate 18: Photomicrograph of testes from group **Stain:** H&E; **Magnification**: x40

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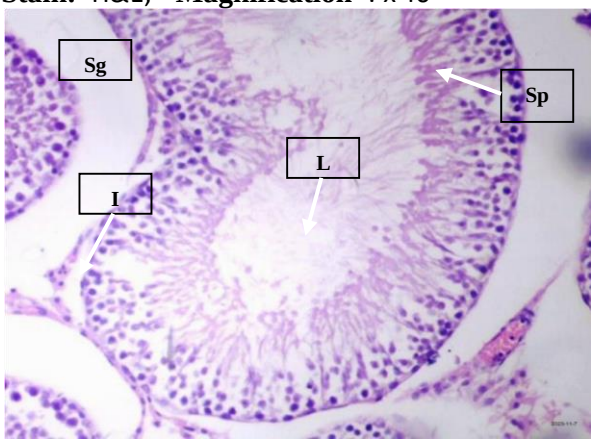
group 7 showing the lumen (L), Spermatozoa 8 showing the lumen (L), Spermatozoa (Sp); (Sp);

Stain: H&E; **Magnification** : x 40



Sertoli Cells (S) and interstitum (I)**.** Sertoli Cells (S) and interstitum (I)**. Stain:**

Plate 19: Photomicrograph of testes from group 9 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S) and interstitum (I)**.** **Stain:** H&E; **Magnification**: x40

H&E; **Magnification** : x 40

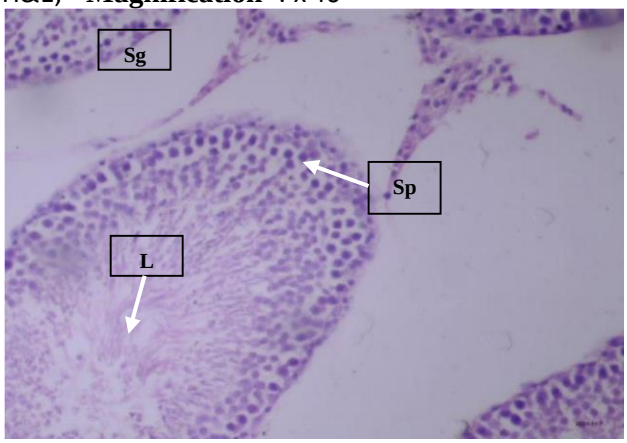


Plate 20: Photomicrograph of testes from group 10 showing the lumen (L), Spermatozoa (Sp); Sertoli Cells (S), and interstitum (I)**. Stain:** H&E; **Magnification**: x40

Discussion

Figures 1 through 3 from this study's analysis of sperm parameters provide important information about how Trichloroacetic Acid (TCA) treatment affects adult male Wistar rats.

In particular, there were notable changes in sperm count and morphology upon the exclusive administration of TCA, with a concurrent increase in aberrant sperm morphology and a considerable decrease in normal sperm morphology ($p \leq 0.05$).

This discovery is consistent with other research, such as the study conducted by Toth *et al*. (1999), which connected prolonged dichloroacetate treatment to sperm count reduction and testicular d egeneration in rats.

In a similar vein, lower fertility and spermatogenesis problems have been linked to brominated a cetic acids, such as dibromoacetic acid (DBAA) and bromochloroacetic acid (BCA), in adult rats.

In particular, rats treated with dichloroacetate at specified dose levels showed significant effects on epididymal sperm counts (Toth *et al*., 1992).

Concerns over TCA's possible impacts on fertility and reproductive health are raised by these dat a, which highlight the drug's detrimental effects on important reproductive parameters. These results are corroborated by histological changes in TCA treated rats, which show impaired spermatogenesis as a result of Leydig cell degeneration and de creased testosterone production (Sanghamitra *et al*., 2008), as well as the detrimental effects of environmental contaminants on testicular function (Akingbemi *et al*., 2004; Murugesan *et al*., 200 7).

By contrast, sperm parameters significantly improved in the groups treated with Eugenol Isolate from Clove (EIC).

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Significant increases in the number of sperm and a decrease in aberrant sperm morphology point to a possible beneficial role for EIC in reducing the harmful effects of TCA on sperm quality. These encouraging results provide hope for preserving reproductive health by suggesting that EI C may be able to partially repair the integrity of sperm parameters in the face of TCA induced difficulties.

Group 6 study yielded an interesting finding: compared to the positive control group, there was a non-significant ($p>0.05$) increase in normal sperm morphology.

This rise, while not statistically significant, points to a possible benefit of the treatment in this particular setting and calls for more research into the complex interactions between the therapies.

Looking closely at the dose-dependent responses reveals that only Groups 7 and 9 showed statistically significant ($p\leq0.05$) changes.

Normal sperm morphology decreased in Group 7, but aberrant sperm morphology changed along with a rise in normal sperm morphology in Group 9.

These particular reactions highlight how crucial dose concerns are to comprehending how therapy affects sperm morphology in this particular experimental setting.

Looking closely at the dose-dependent responses reveals that only Groups 7 and 9 showed statistically significant ($p\leq0.05$) changes.

Normal sperm morphology decreased in Group 7, but aberrant sperm morphology changed along with a rise in normal sperm morphology in Group 9.

These particular reactions highlight how crucial dose concerns are to comprehending how therapy affects sperm morphology in this particular experimental setting.

In summary, the collective results from figures 5 and 6 shed light on the intricate interplay between TCA, EIC, and sperm morphology. These findings underscore the potential for both detrimental and beneficial effects on various sperm parameters, prompting critical questions about the complex dynamics at play and their broader implications for reproductive health.

This study revealed significant disparities between the positive and negative control groups. The positive control group exhibited a noteworthy increase in malondialdehyde (MDA) levels and a marked decline in catalase (CAT) and superoxide dismutase (SOD) levels compared to the negative control group, indicating a substantial alteration in oxidative stress indicators. This outcome aligns with previous research on TCA, where oxidative stress indicators accumulated in response to dichloroacetate injection (Calcutt *et al.*, 2009), and TCA exposure led to lipid peroxidation and alterations in antioxidant systems in rats (Çelik, 2007).

Studies by Austin *et al*. (1996) demonstrated a surge in TCA-induced thiobarbituric acid-reactive substances (TBARS), indicative of lipid peroxidation, in mice livers nine hours after administration.

Numerous investigations have explored the potential of Trichloroacetic Acid (TCA) to induce oxidative stress and peroxisome proliferation. The detoxification of Reactive Oxygen Species (ROS) involves antioxidant enzymes, constituting a crucial aspect of the immune system. The primary defense against oxygen-related damage is the SOD–CAT system, where Superoxide Dismutase (SOD) plays a pivotal role in converting superoxide anions to hydrogen peroxide (H2O2) during oxidative stress (Halliwell & Gutteridge, 1989). H_2O_2 elimination is subsequently managed by either Catalase (CAT) or Glutathione Peroxidase (GPx), with GPx predominantly operating in the testes (Peltola *et al*., 1992). Increased SOD activity may result from heightened superoxide anion

generation, indicating an adaptive response to combat free radicals, with a subsequent rise in CAT activity (Cheung *et al*., 2001; Braga *et al*., 2009).

Conversely, elevated levels of ROS pose a threat to testicular function. The testes possess a robust antioxidant system, including the glutathione family, superoxide dismutase, catalase, and nonenzymatic antioxidants, shielding against the harmful effects of ROS (Aitken & Roman, 2008).

Nevertheless, excessive exposure to environmental toxins has been shown to disturb the prooxidant/antioxidant equilibrium in the testes, impairing their function (Saradha & Mathur, 2006).

It is noteworthy however, that various studies have explored TCA-induced oxidative stress responses in mice, including lipid peroxidation and oxidative DNA damage, with temporary increases observed in single-dose trials. Conversely, peroxisome proliferation-related reactions persisted for up to 10 weeks after TCA dosage. Findings of the current study indicates a consistent pattern among treatment groups, specifically groups 6 through 8, showing reduced MDA levels, indicative of lowered lipid peroxidation, and increased SOD and CAT levels, suggesting improved antioxidant defenses compared to the positive control group. Interestingly, the combination of higher doses of Eugenol Isolates from Clove (EIC) with TCA exhibited synergistic effects, leading to a more pronounced reduction in SOD levels and a concurrent decrease in MDA levels, suggesting a potent mitigation of lipid peroxidation. This underscores the potential dose-dependent synergies between EIC and TCA in reducing oxidative stress.

Histological examination of testicular tissues in groups 1-3 revealed a standard histological profile. This profile was characterized by an abundance of spermatozoa extending towards the lumen, intact Leydig cells, and noticeable spermatid retention within the seminiferous tubules. In stark contrast, groups 4 and 5 exhibited abnormal seminiferous tubule morphology, marked by spermatid retention, tubular atrophy, and widespread disorganization of germ cells. The testicular architecture displayed signs of degeneration, evident in the absence of interstitial space and areas of necrosis. Notably, maturing spermatogenic cells were identified within the seminiferous tubules, some displaying nuclear membrane rupture and nucleus fragmentation (karyorrhexis).

In a study conducted by Singh (2005), a comprehensive analysis involving dissection, weight measurement, and histological examination was carried out on the testes of pups from various dose groups. Fetal testes exhibited a significant reduction in average weights, particularly conspicuous at doses equal to or exceeding 1,200 mg/kg-day when contrasted with the control group. Histological examination of fetal rat testes within the 1,200 mg/kg-day dose group revealed a distinct pattern; there was a noticeable reduction in the diameter of seminiferous tubules, predominantly confined to the peripheral region. This effect became more pronounced in groups receiving higher doses, accompanied by a reported reduction in the length of seminiferous tubules at elevated dose levels.

On closer inspection at higher magnification, a compelling finding emerged: an elevated occurrence of apoptosis in both gonocytes and Sertoli cells within the seminiferous tubules, particularly evident in comparison to the controls at doses equal to or exceeding 1,200 mg/kg-day. This detailed histological analysis sheds light on the nuanced impact of varying doses on testicular morphology, emphasizing the importance of considering both dosage levels and specific histopathological alterations in understanding the observed effects.

Conversely, groups 6-10 presented distinctive characteristics, featuring spermatogonia cells with deeply stained nuclei and damaged sperm cells. The seminiferous tubules in these groups displayed a shrunken appearance with

a wavy outline. Thickening and hyalinization of basement membranes were apparent, and the lumens of the seminiferous tubules were predominantly occupied by fragments of disintegrated cells. While sharing some similarities with groups 4 and 5, the histological features of groups 6-10 stand apart, preventing a direct comparison to the observations in groups 1-3.

Eugenol, a key component in clove oil, demonstrates notable therapeutic potential across various physiological domains, as evidenced by multiple studies. Damiani *et al*. (2003) found that Eugenol induces smooth muscle relaxation in rats by blocking voltage-sensitive and receptor-operated channels modulated by endothelial-generated nitric oxide. Al-Okbi *et al*. (2014) demonstrated that clove essential oil and Eugenol micro emulsions provide significant improvement in fatty liver and dyslipidemia in rats, offering protection against cardiovascular diseases and complications associated with fatty liver.

The current study provides comprehensive insights into the modulating effects of clove isolates, particularly Eugenol Isolates from Clove (EIC), on testicular pathohistological changes and spermredox status in Trichloroacetic Acid (TCA)-treated rats. The examination of sperm parameters highlighted the detrimental impact of TCA on sperm count and morphology, consistent with previous research associating TCA exposure with testicular degeneration and reduced sperm counts. Histological alterations further supported these findings, emphasizing disrupted spermatogenesis and Leydig cell degeneration induced by TCA.

In conclusion, eugenol; a key component in clove oil, has demonstrated therapeutic potential in various physiological domains, adding to the significance of our findings. Overall, our study emphasizes the intricate interplay between TCA, EIC, and reproductive parameters, providing a foundation for further research and suggesting potential avenues for therapeutic interventions to safeguard testicular health in the face of environmental challenges.

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