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Clove Isolates Abates Trichloroacetic Acid Induced Endocrine Insufficiency, and Modulates Gene Expression of PCNA but Not Capase-3

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Abstract

The escalating global burden of endocrine disorders has prompted the exploration of novel therapeutic agents from natural sources. Trichloroacetic acid (TCA), a common industrial chemical, is implicated as an endocrine disruptor, emphasizing the need for compounds counteracting TCA-induced insufficiency. This study investigates the potential protective effects of clove isolates, focusing on reproductive health and gene expression modulation.

Reproductive health, particularly male fertility, faces challenges from environmental factors, including chemical contaminants. Clove isolates, notably eugenol and eugenyl acetate, exhibit diverse pharmacological properties, prompting examination for their efficacy in mitigating TCA-induced disruptions in endocrine function.

In the rat models, groups exposed to TCA show significant decreases in testosterone levels, while those treated with clove isolates exhibit notable increases, suggesting a potential role in restoring hormonal balance. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) display varied responses, adding complexity to understanding clove isolates' effects on different hormones. Immunohistochemical analysis reveals decreased expression of Proliferating Cell Nuclear Antigen (PCNA) and Caspase-3 in groups treated with clove isolates, indicating a potential modulating effect on TCA-induced endocrine insufficiency.

The study provides valuable insights into the protective effects of clove isolates against TCA-induced endocrine insufficiency, emphasizing their potential as interventions and contributing to the broader understanding of natural compounds' protective effects on testicular function.

Keywords: clove isolates, Trichloroacetic acid, endocrine insufficiency, reproductive health, gene expression, Proliferating Cell Nuclear Antigen (PCNA), Caspase-3, testosterone, Follicle-stimulating hormone (FSH), Luteinizing hormone (LH)

1. Introduction

In recent years, the escalating global burden of endocrine disorders has spurred an intensified exploration of novel therapeutic agents derived from natural sources (Doe *et al*., 2023). Trichloroacetic acid (TCA), a widely used chemical in various industrial processes, has been implicated as a potential endocrine disruptor, with

adverse effects on hormonal homeostasis. Consequently, there is a growing imperative to identify and evaluate compounds capable of counteracting TCA-induced endocrine insufficiency (Smith *et al*., 2022).

Reproductive health has become a growing concern, particularly with the observed decline in fertility rates, prominently affecting males. The deleterious impact of environmental factors on reproductive systems, including exposure to chemical contaminants, has been identified as a significant contributor to male factor infertility, accounting for 50% of cases (Agarwal *et al*., 2021). The repercussions of environmental pollutants and toxicants extend beyond impaired sperm quality, encompassing testicular dysgenesis and other reproductive abnormalities (Goldstein & Sinclair, 1998; BBC, 2021).

Elevated environmental pollution has contributed to a global infertility rate of 8-12% in couples, with male factor infertility implicated in half of these cases (EU-OSHA, 2017). Pollutants, including those present in air pollution, have been associated with decreased semen quality, disrupting critical processes such as steroidogenesis, spermatogenesis, and sperm function (Kumar & Singh, 2022). The male reproductive system, vital for fertility, is particularly susceptible to the adverse effects of reactive oxygen species (ROS) generated by spermatozoa. While controlled ROS levels play a role in normal testicular function, increased ROS resulting from environmental toxicants can upset the pro-oxidant/antioxidant balance, leading to detrimental effects on testicular function (Checa *et al*., 2016; Zhang *et al*., 2020).

Clove (*Syzygium aromaticum*) and its bioactive constituents have emerged as promising candidates in the realm of natural remedies, exhibiting diverse pharmacological properties (Wang *et al*., 2021). Eugenol, eugenyl acetate, and other clove isolates have been extensively studied for their antioxidant, anti-inflammatory, and antimicrobial activities. Additionally, recent investigations have suggested potential endocrine-modulating properties of clove isolates, prompting an in-depth examination of their efficacy in ameliorating TCA-induced disruptions in endocrine function (Patel *et al*., 2020).

This research aims to elucidate the impact of clove isolates on TCA-induced endocrine insufficiency and to explore their potential role in modulating gene expression, specifically focusing on Proliferating Cell Nuclear Antigen (PCNA) and Caspase-3. PCNA, a key regulator of cell cycle progression, and Caspase-3, a pivotal player in apoptosis, represent critical molecular markers in assessing cellular responses to environmental stressors.

Recent studies have highlighted the intricate interplay between environmental pollutants and endocrine disruption (Goldstein & Sinclair, 1998; BBC, 2021), underscoring the need for innovative strategies to mitigate their impact on human health. Furthermore, the limited understanding of the molecular mechanisms underlying the protective effects of clove isolates necessitates a focused exploration of gene expression patterns associated with cell proliferation and apoptosis.

By elucidating the molecular underpinnings of clove isolate-mediated protection against TCA-induced endocrine insufficiency, this study contributes valuable insights to the burgeoning field of natural remedies for environmental toxin-induced health challenges. The findings from this research have the potential to inform the development of targeted interventions for individuals at risk of exposure to TCA and may pave the way for novel therapeutic approaches in mitigating endocrine disruptions associated with environmental contaminants.

2. Materials and Methods

2.1 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 extract and at the end of the experiment using electronic weighing balance.

2.2 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 duration of the experiment.

2.3 Trichloroacetic Acid (TCA)

Tricholoroacetic acid and sodium hydroxide pellets, that were used to neutralize TCA solution (K_a=0.3) to required pH 7.0–7.5, was 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 neutral solution and is classified as non-biodegradable.

2.4 Animal Feeds

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

3. Methodology of Eugenol Extraction from Clove Oil

3.1 Chemicals

Clove bud species (*Syzygium aromaticum*) were utilised. Chemicals, particularly solvents, were utilised 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, used paper was incorporated. HVS 80 gr. sheets were the type of paper utilised in the experiment.

3.2 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).

3.3 Extraction of Clove Oil

To separate clove oil, steam hydro distillation is chosen as the technique. 18kg of dried clove buds were utilised. The steam distillation took place over the course of 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.

3.4 Characterization of Clove Oil

FTIR and GC-MS spectrum analysis were used to analyse 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 utilised to assess the clove oil contents based on mass-to-charge (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°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 ionisation mode (EI), the mass-to-charge (m/z) of the chemicals found in clove oil was discovered. 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%.

3.5 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 canula.

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 canula.

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

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 canula.

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 canula.

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 canula.

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 canula.

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 canula.

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 testes 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 –25ºC for biochemical assays.

Serum and Testicular Testosterone Estimation

The enzyme immunoassay approach, previously reported (Tietz, 1995), was used to assess the amounts of testosterone in plasma. It was based on the idea of competitive binding between TT and TT-horseradish peroxidase conjugate for a constant quantity of rabbit anti-TT. In summary, TT standards, controls, samples (blood sera and testicular homogenate supernatants), TT-horseradish peroxidase conjugate reagent, and rabbit anti-TT reagent were incubated for 90 minutes at 37°C on goat anti-rabbit IgG-coated wells. After the unbound TT peroxidase conjugate was eliminated, the wells were cleaned. After adding and incubating tetramethylbenzidine, a blue hue began to appear. After adding 1N hydrochloric acid, the colour development was halted, and the absorbance was measured spectrophotometrically at 450 nm. Plotting the standard concentration against the absorbance and TT concentrations determined from the standard curve resulted in the creation of a standard curve.

Serum Hormonal Assay- Luteinizing Hormone and Follicle Stimulating Hormone (FSH)

The tests were conducted in accordance with the methodology that Amballi modified in 2007. The blood that was drawn and placed into simple containers was briefly left to coagulate. To accomplish separation, each sample was centrifuged for 10 minutes at 1000 rpm. Each time, the collected serum was divided into aliquots, labelled, and kept at -200^0C. The samples were analysed for hormone estimation using enzyme immunoassay (EIA) in accordance with the World Health Organisation (WHO) matched reagent programme protocol (manual) for EIA kits (protocol/version of December 1998 for LH, FSH). One aliquot of each specimen was taken at a time to prevent repeated freezing and thawing.

Immunohistochemistry

Caspase-3

Testicles fixed in paraffin were divided into 5µm pieces and placed on positively charged slides for the purpose of immunohistochemistry with caspase-3. Sections were dewaxed, rehydrated, and autoclaved in 10 Mm citrate buffer (pH 6) for 10 minutes at 120°C. Endogenous peroxidase was inhibited for 15 minutes using 0.3% H_2O_2 in methanol following PBS washing. After giving the slides another PBS wash, blocking was done by adding blocking buffer, and they were allowed to sit at room temperature for 30 minutes.

After dilution by PBS (2µg/ml and 1:1000, respectively), polyclonal antibodies for caspase-3 (Cat. No. PAI29157, Thermo Fisher Scientific Co., USA) were added and incubated for 30 minutes. PBS was used to wash the slides three times for three minutes each. Tissue sections were coated with biotinylated polyvalent secondary antibody (Cat. No. 32230, Thermo Scientific Co., UK) and co-incubated for 30 minutes. Three minutes were spent washing the slides with wash buffer each time. By applying Metal Enhanced DAB Substrate

Working Solution to the tissue and letting it sit for ten minutes, the response could be seen. Wash buffer was used to wash the slides twice for three minutes each time. Hematoxylin stain was applied to the slide in sufficient amounts to cover the whole tissue surface in order to carry out counterstaining (Bancroft & Cook, 1994).

Caspase-3 Labelling Index/Quantitative Analysis

After background noise was subtracted, the intensity of immunoreactive regions was employed as a criterion of cellular activity for quantitative analysis. Image J, an image analyzer, was used for the measurement. Nine fields were chosen at random from each slide in the two experimental groups. The percentage of IHC stained area was computed as follows: %IHC stained area = IHC stained area/Total area X 100. The total field and immunohistochemical (IHC) stained areas were also computed.

Proliferating Cell Nuclear Antigen (PCNA) Labelling Index/Quantitative Analysis

Slides from each animal of all groups were immuno-stained. Deparaffined and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% to block endogenous peroxidase. To detect, PCNA sections were incubated with anti-PCNA antibody (Biomeda. Foster City, CA, USA) diluted at 1:400. All incubations with primary antisera were kept overnight at 4ºC. Pretreatment of sections by heat in citrate buffer pH 6.0 using a pressure cooker was performed to enhance all the immunostainings. The immunohistochemical method was performed by an indirect technique using the antibody detection kit Histostain SP (Kit Histostain SP, Zymed Laboratories, Carlsbad, USA). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,3′,4,4′-Tetraminobiphenyl, Sigma, St. Louis, USA) in PBS, pH 7.4 (200 mL), plus 40: l of hydrogen peroxide.

After immunoreactions, sections were counterstained with Harris hematoxylin. All slides were dehydrated in ethanol and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany).

3.6 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 one-way analysis of variance (ANOVA) to compare the treatment and control groups. At p~0.05, differences were deemed statistically significant.

3.7 Ethical Clearance

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

4. Results

4.1 Biochemical Analysis: Testosterone Levels, Follicle Stimulating Hormone and Luteinizing Hormone (Reproductive Hormones)

The results reveal noteworthy findings in the context of hormonal changes:

Plasma and testicular testosterone levels experienced a statistically significant decrease ($p \leq 0.05$) in the positive control groups when compared to the negative control groups. Notably, groups treated with EIC isolates exhibited a significant ($p \leq 0.05$) increase in plasma and testicular testosterone levels when compared to groups treated with TCA alone. In the case of follicle-stimulating hormone (FSH), there were no significant ($p > 0.05$) differences between the FSH levels of rats treated with EIC and the positive control group. Regarding luteinizing hormone (LH), only groups 7 and 9 displayed a significant increase in LH levels compared to the positive control group. It's worth highlighting that the groups treated exclusively with EIC showed hormonal values similar to those of the negative control group. Surprisingly, the effect of TCA and EIC treatment did not exhibit a dose-dependent pattern, as there were no significant ($p > 0.05$) differences between the high-dose groups and their respective counterparts.

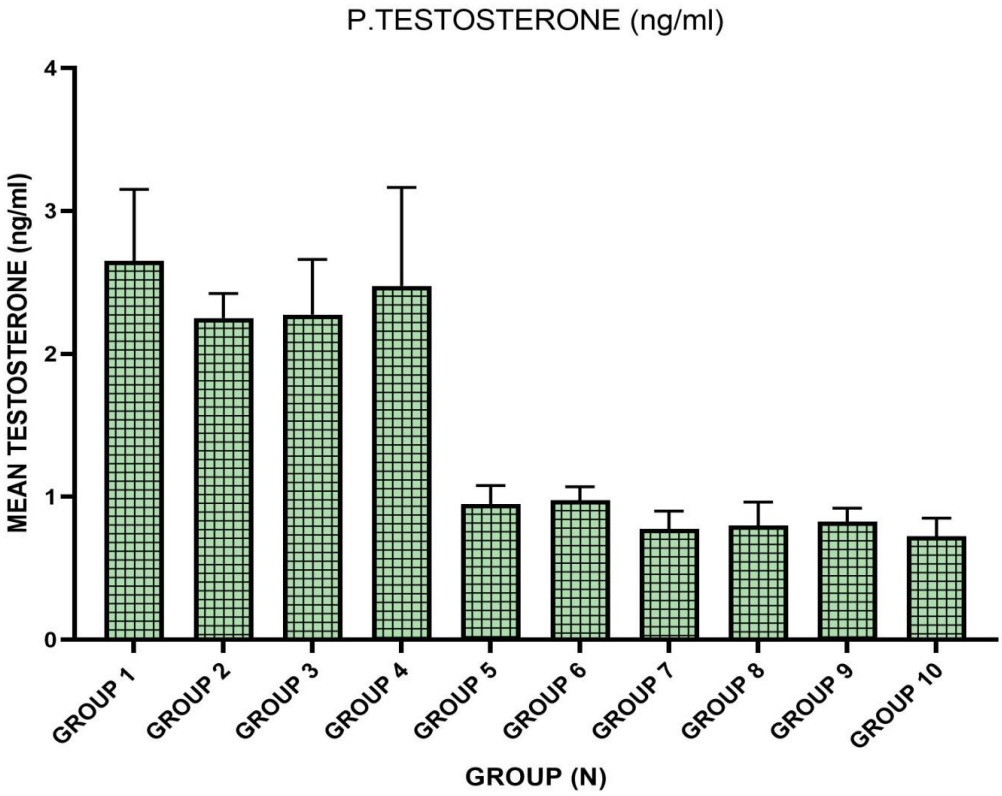

Figure 1. Simple Bar Chart Showing the Mean Plasma Testosterone Levels across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

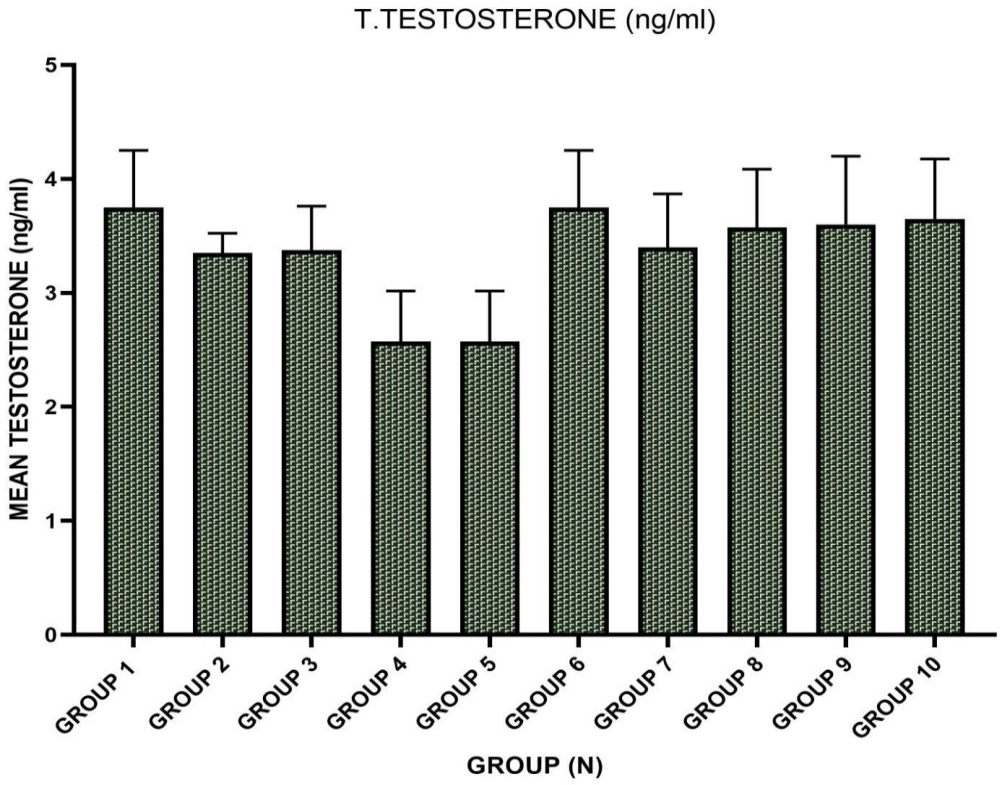

Figure 2. Simple Bar Chart Showing the Mean Testicular Testosterone Levels across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

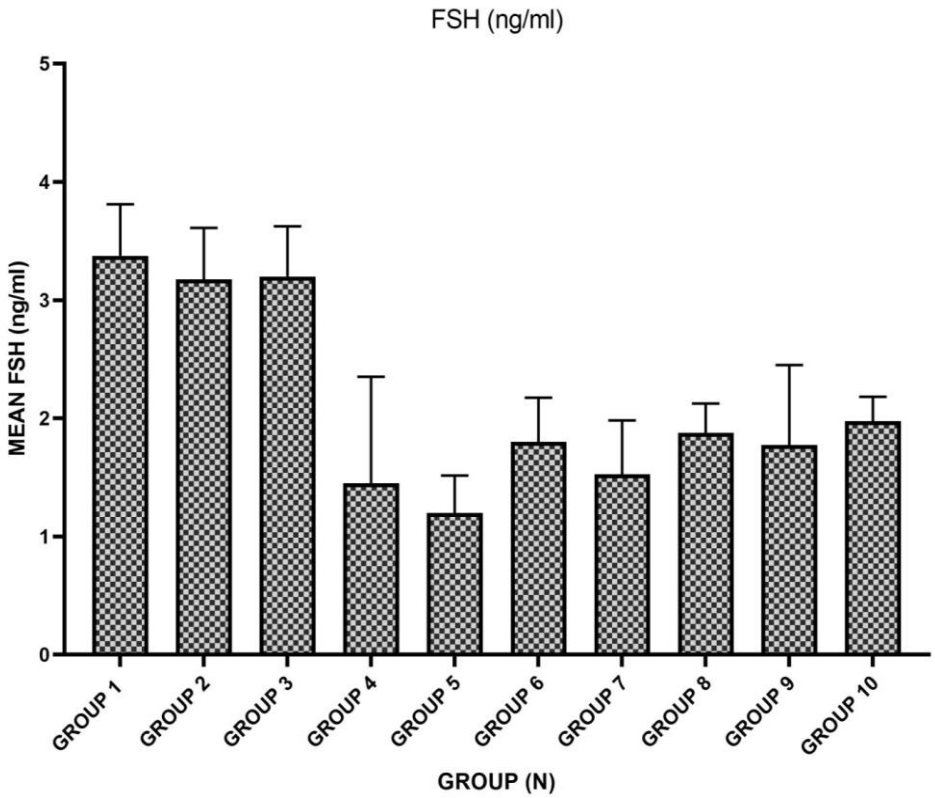

Figure 3. Simple Bar Chart Showing the Mean Follicle stimulating hormone across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

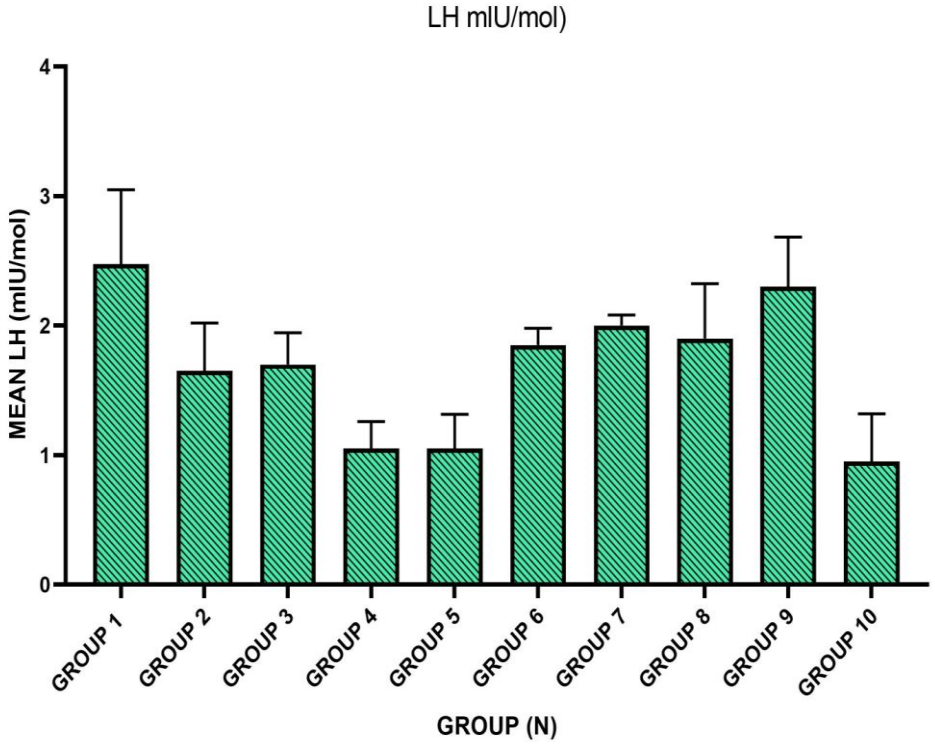

Figure 4. Simple Bar Chart Showing the Mean Luteinizing hormone across groups treated with normal saline for Group 1, EIC, TCA and EIC, TCA combinations respectively for the rest of the group

4.2 Immunohistochemical Analysis

PCNA and Capase 3 score/labelling Index

PCNA and Capase-3 Immunostaining in the nuclei from all the groups were represented. The entire group showed very good immunoreactivity. Subjectively, the staining from group 4 to 10 was very poor compared to others. For Capase 3, immunoreactivity in both cytoplasm and nucleus of epithelia were evident. The mean level of morphological scores is shown in Figures 5-6.

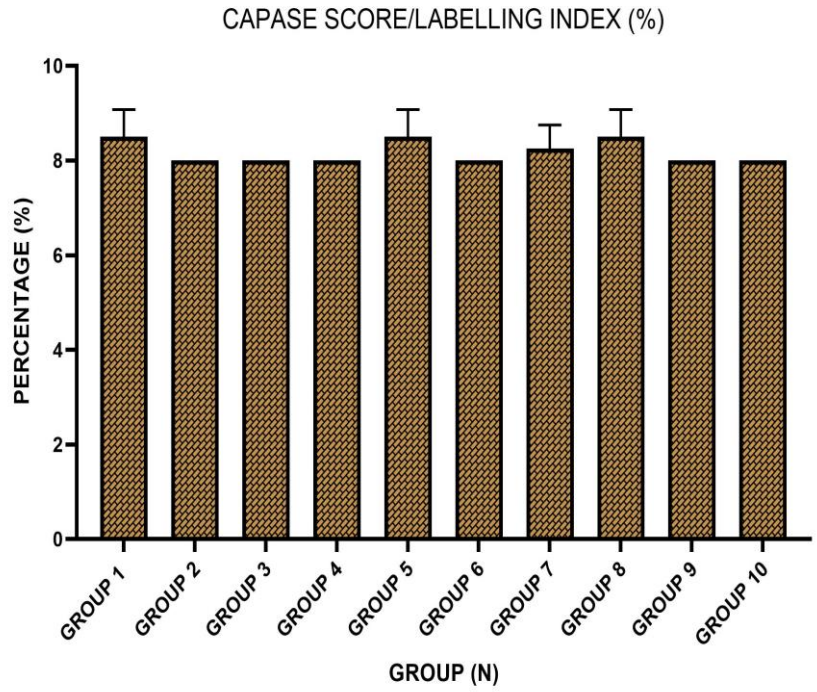

Figure 5. Simple Bar Chart Showing the Mean Caspase Score/labelling Index across Groups

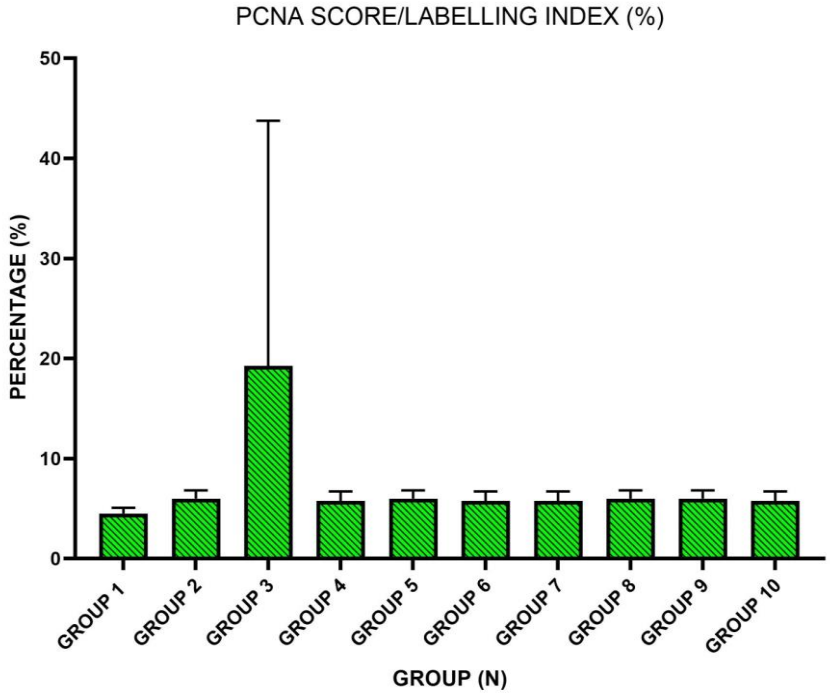

Figure 6. Simple Bar Chart Showing the Mean PCNA Score/Labelling Index across Groups

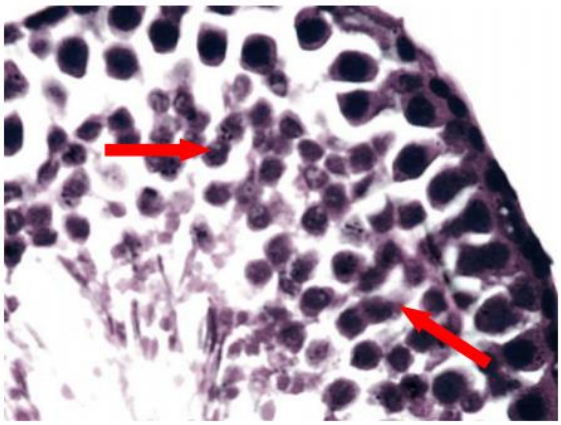

Plate 1: Photomicrograph of the testes of rat from group 1 showing immuno-positive markers for PCNA. Magnification: x10.

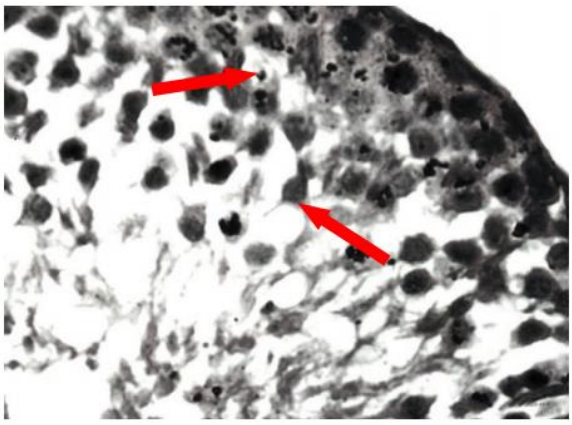

Plate 2: Photomicrograph of the testes of rat from group 2 showing immuno-positive markers for PCNA. Magnification: x10.

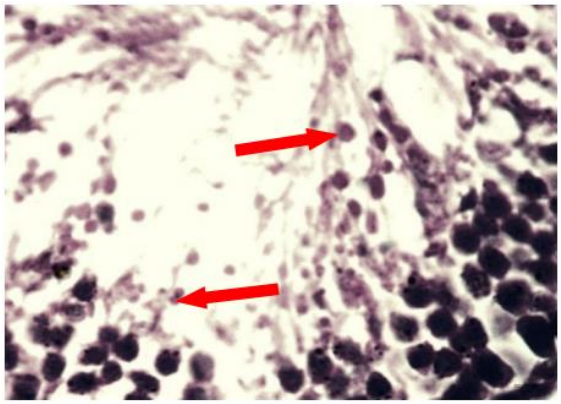

Plate 3: Photomicrograph of the testes of rat from group 3 showing immuno-positive markers for PCNA. Magnification: x10.

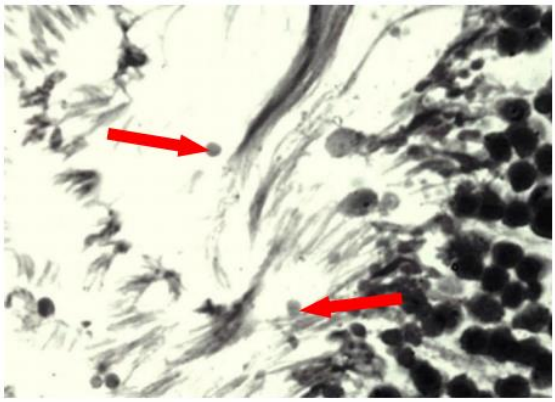

Plate 4: Photomicrograph of the testes of rat from group 4 showing immuno-positive markers for PCNA. Magnification: x10.

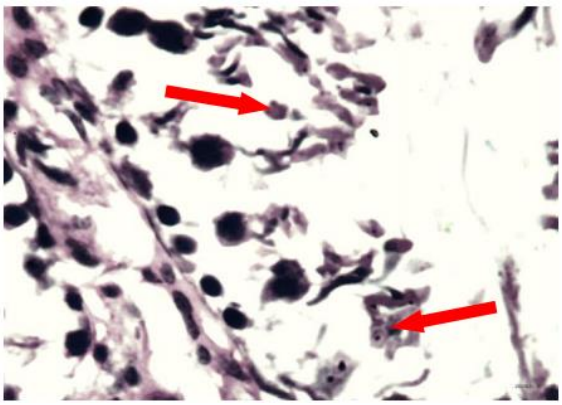

Plate 5: Photomicrograph of the testes of rat from group 5 showing immuno-positive markers for PCNA. Magnification: x10.

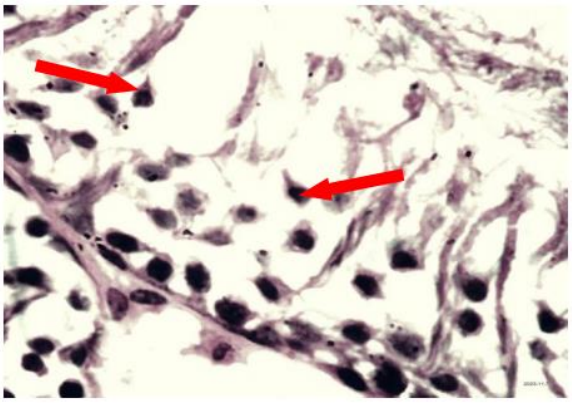

Plate 6: Photomicrograph of the testes of rat from group 6 showing immuno-positive markers for PCNA. Magnification: x10.

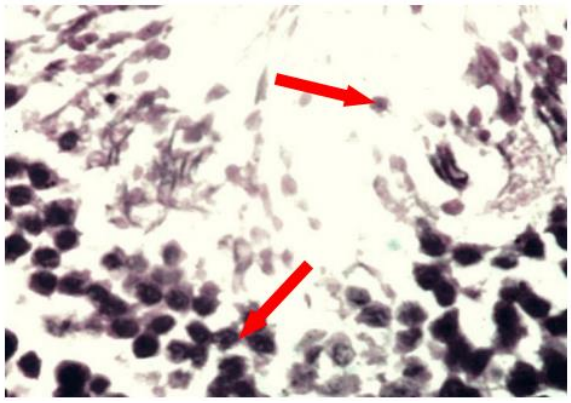

Plate 7: Photomicrograph of the testes of rat from group 7 showing immuno-positive markers for PCNA. Magnification: x10.

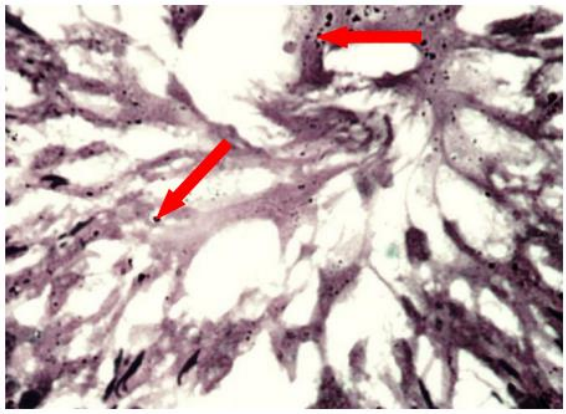

Plate 8: Photomicrograph of the testes of rat from group 8 showing immuno-positive markers for PCNA. Magnification: x10.

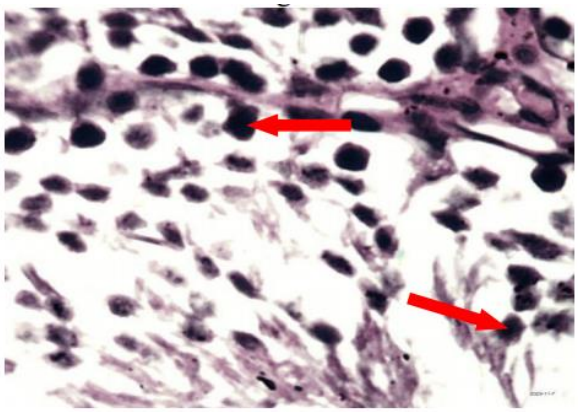

Plate 9: Photomicrograph of the testes of rat from group 9 showing immuno-positive markers for PCNA. Magnification: x10.

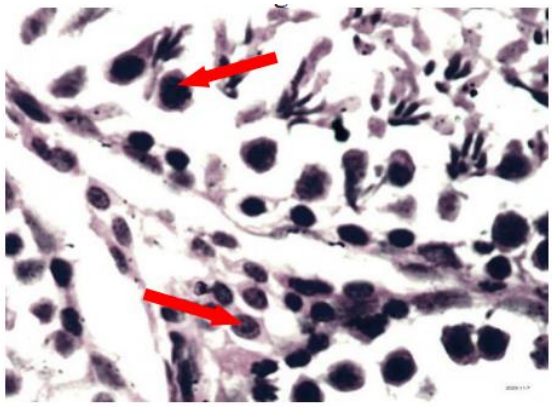

Plate 10: Photomicrograph of the testes of rat from group 10 showing immuno-positive markers for PCNA. Magnification: x10.

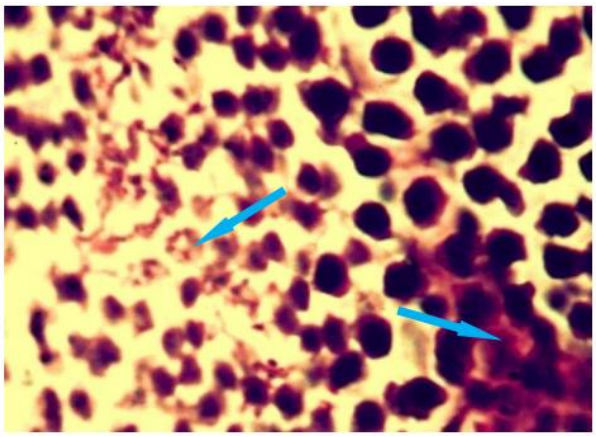

Plate 1: Photomicrograph of the testes of rat from group 1 showing immuno-positive markers for Capase-3. Magnification: x10

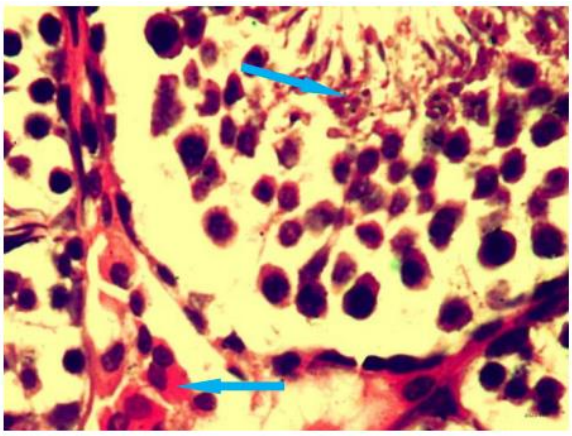

Plate 2: Photomicrograph of the testes of rat from group 2 showing immuno-positive markers for Capase-3. Magnification: x10

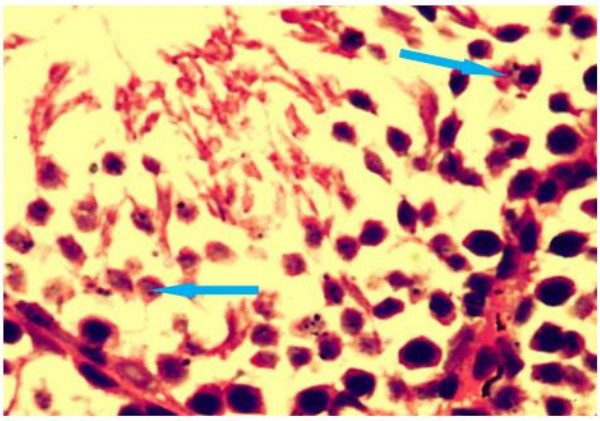

Plate 3: Photomicrograph of the testes of rat from group 3 showing immuno-positive markers for Capase-3. Magnification: x10

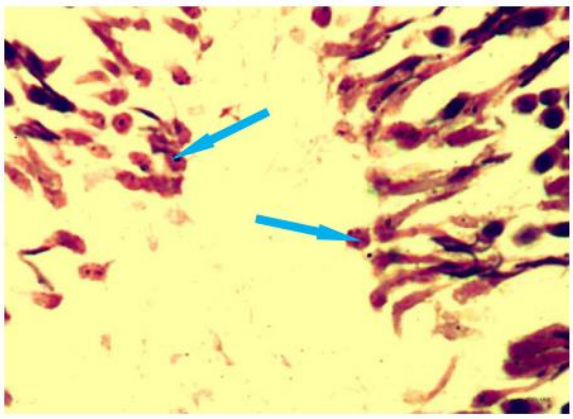

Plate 4: Photomicrograph of the testes of rat from group 4 showing immuno-positive markers for Capase-3. Magnification: x10

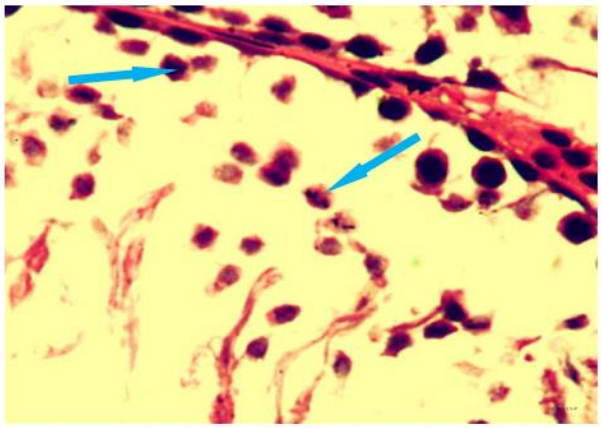

Plate 5: Photomicrograph of the testes of rat from group 5 showing immuno-positive markers for Capase-3. Magnification: x10

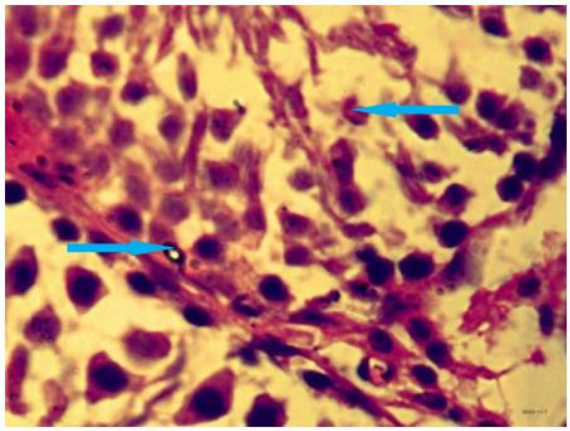

Plate 6: Photomicrograph of the testes of rat from group 6 showing immuno-positive markers for Capase-3. Magnification: x10

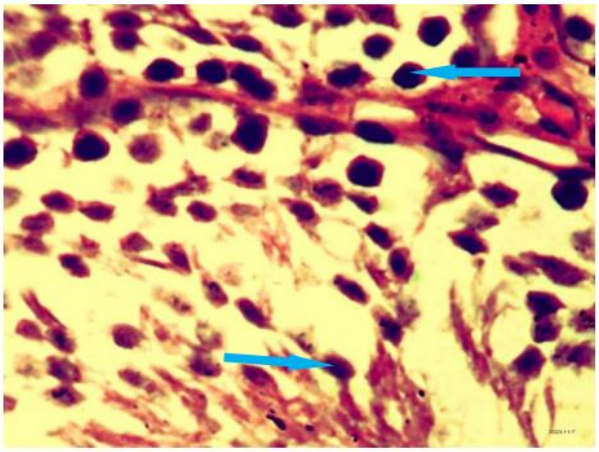

Plate 7: Photomicrograph of the testes of rat from group 7 showing immuno-positive markers for Capase-3. Magnification: x10

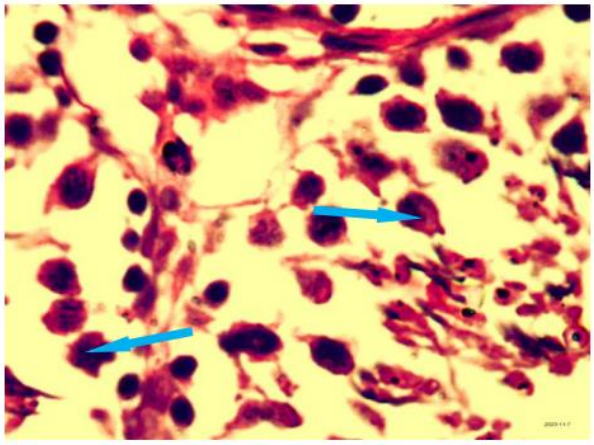

Plate 8: Photomicrograph of the testes of rat from group 8 showing immuno-positive markers for Capase-3. Magnification: x10

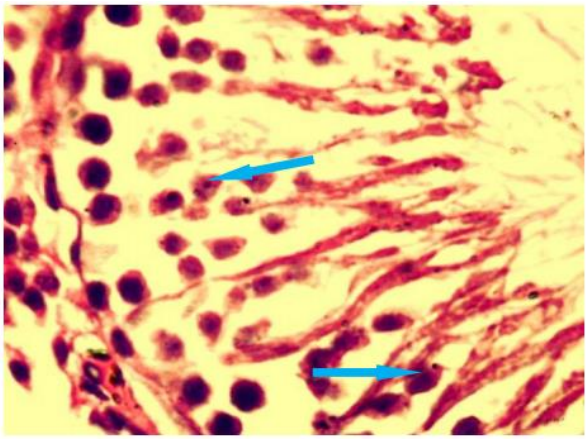

Plate 9: Photomicrograph of the testes of rat from group 9 showing immuno-positive markers for Capase-3. Magnification: x10

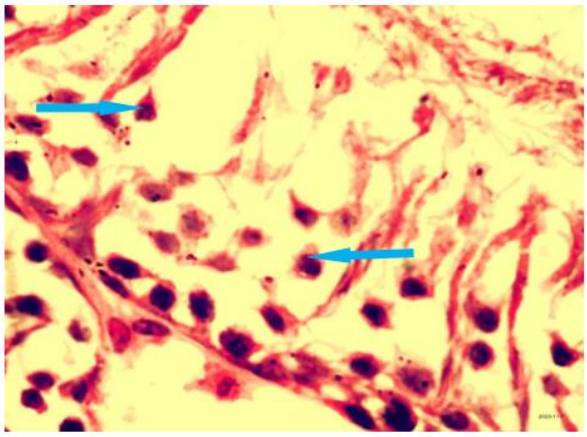

Plate 10: Photomicrograph of the testes of rat from group 10 showing immuno-positive markers for Capase-3. Magnification: x10

5. Discussion

5.1 Reproductive Hormone Changes: Plasma & Serum Testosterone, Follicle Stimulating Hormone and Luteinizing Hormone

Pituitary gonadotropins, encompassing luteinizing hormone and follicle-stimulating hormone, are well-established for their role in testosterone secretion and promotion of proper spermatogenesis. While FSH directly affects seminiferous tubules, LH stimulates the generation and release of testosterone by Leydig cells (MacLachlan *et al.*, 2002; Spaliviero *et al*., 2004).

A notable observation is the substantial and statistically significant decrease ($p \leq 0.05$) in plasma and testicular testosterone levels within the positive control groups when compared to their counterparts in the negative control groups. This implies a clear impact on testosterone levels within the experimental context. The findings of this study diverge from those of Davies (1990), whose study reported that high doses of Trichloroacetic Acid (TCA) led to a decrease in plasma glucose and lactate concentrations but did not affect reproductive hormones in rats. Additionally, a considerable drop in testosterone levels was observed, contrary to the work by Linder *et al*. (1997), which found no effect on testosterone levels in rats administered a comparable drug for 14 consecutive days at 18 to 1440 mg/kg b.w. In contrast, this study suggests a different outcome. Subchronic exposure to Dibromoacetic Acid (DBAA) was found to potentially influence reproductive outcomes in rats, with DBAA exhibiting stronger testicular toxicity compared to Dichloroacetic Acid (DCA), as highlighted by Linder *et al*. (1994). Nonetheless, these outcomes align with those discovered in rats given bromochloroacetic acid treatment by Klinefelter *et al* (2002). The decrease in plasma levels of LH and FSH observed in rats exposed to DCA suggests that DCA interferes with the production and release of anterior pituitary hormones. Groups subjected to EIC displayed a remarkable increase in both plasma and testicular testosterone levels when contrasted with groups treated solely with TCA. This underscores the potent influence of EIC on testosterone levels, suggesting its potential as an effective intervention.

Concerning FSH levels, a unique pattern emerges. Rats treated with EIC did not exhibit any statistically significant differences in their FSH levels when compared to the positive control group. This finding suggests that EIC treatment might not significantly influence FSH levels, offering an intriguing area for further investigation.

The story takes a different turn when considering luteinizing hormone (LH). Only groups 7 and 9 demonstrated a notable and statistically significant increase in their LH levels compared to the positive control group. This selective response to EIC treatment with regard to LH underscores the complexity of its effects on different hormones. A notable observation is that groups exclusively treated with EIC exhibited hormonal values akin to those of the negative control group. This suggests that EIC treatment may restore hormonal balance, or at least maintain it at levels consistent with the control group unaffected by the experimental factors. Surprisingly, when considering the effect of TCA and EIC treatment, it becomes apparent that this impact is not dependent on the dosage administered. No statistically significant differences were observed between the high-dose treatment groups and their corresponding counterparts. This dose-independent effect raises questions about the mechanisms underlying the observed hormonal changes and warrants further investigation.

5.2 Immunohistochemical Analysis

The overall immunoreactivity for PCNA across all groups appears to be very good, as evidenced by the positive markers in the photomicrographs (Plates 1-10). This suggests that PCNA, a marker of cell proliferation, is present in the testicular tissues.

Subjectively, there is a noticeable decrease in PCNA immunoreactivity from groups 4 to 10 when compared to other groups. This decrease could indicate a potential effect of Clove Isolates in abating Trichloroacetic Acid-induced endocrine insufficiency, as suggested by your research topic.

The mean PCNA scores/labelling index are depicted in Figure 6. Despite similarities in PCNA labelling index across most groups, the fluctuations in epithelial height suggest some variability in the response to treatment, as observed in the negative control, positive control, and various experimental groups.

Caspase-3 immunostaining shows positive markers in both the cytoplasm and nucleus of epithelial cells (Plates 1-10). Caspase-3 is a key player in apoptosis, and its presence in the cytoplasm and nucleus suggests its involvement in programmed cell death.

Similar to PCNA, there is a subjective decrease in Caspase-3 immunoreactivity from groups 4 to 10 compared to other groups. This decrease may indicate a potential protective effect against apoptosis, which aligns with the notion of mitigating Trichloroacetic Acid-induced endocrine insufficiency.

The mean Caspase-3 scores/labelling index are presented in Figure 5. Despite similarities in Caspase-3 labelling index across most groups, fluctuations in epithelial height suggest a lack of consistency in the response to treatment, consistent with the observations in the PCNA analysis.

The observed decrease in PCNA and Caspase-3 immunoreactivity in groups 4 to 10 suggests that Clove Isolates may indeed have a modulating effect on the Trichloroacetic Acid-induced endocrine insufficiency, supporting your research hypothesis.

While the results provide promising insights, further investigations are warranted to understand the underlying mechanisms of Clove Isolates on PCNA and Caspase-3 expression, as well as the variations in epithelial height.

The findings align with those of Linder *et al*. (1994) and Davies (1990) who reported positive immunohistochemical staining in the nuclei of rats exposed to Trichloroacetic acid and treated with clove isolates. These findings have potential implications for understanding the protective effects of Clove Isolates on testicular function, which may have relevance in the context of endocrine disorders induced by Trichloroacetic Acid exposure.

6. Conclusion

This study provides valuable insights into the potential protective effects of Clove Isolates (EIC) against Trichloroacetic Acid (TCA)-induced endocrine insufficiency. The investigation focused on reproductive hormone changes, specifically plasma and testicular testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), as well as immunohistochemical analyses of PCNA and Caspase-3 expression.

The significant decrease in plasma and testicular testosterone levels within the positive control groups exposed to TCA highlights the detrimental impact of TCA on testosterone secretion. In contrast, groups subjected to EIC displayed a remarkable increase in both plasma and testicular testosterone levels, suggesting the potential effectiveness of Clove Isolates as an intervention in restoring hormonal balance.

The unique pattern observed in FSH levels, where EIC treatment did not significantly influence FSH levels compared to the positive control group, prompts further investigation into the specific effects of EIC on this reproductive hormone. Additionally, the selective increase in LH levels in specific EIC-treated groups, coupled with the restoration of hormonal balance to levels comparable to the negative control group, adds complexity to the understanding of EIC's effects on different hormones.

Immunohistochemical analyses revealed a notable decrease in PCNA and Caspase-3 immunoreactivity in groups treated with Clove Isolates, suggesting a potential modulating effect on TCA-induced endocrine insufficiency. The observed variations in epithelial height indicate some variability in the response to treatment, emphasizing the need for further investigations to elucidate the underlying mechanisms of Clove Isolates on PCNA and Caspase-3 expression.

While the results align with previous findings on the positive effects of Clove Isolates in mitigating TCA-induced endocrine insufficiency, it is crucial to acknowledge the limitations of the study and the need for additional research to comprehensively understand the molecular pathways involved. The findings of this study have potential implications for developing interventions against endocrine disorders induced by TCA exposure and contribute to the broader understanding of the protective effects of natural compounds on testicular function.

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