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Quercetin-Mediated Modulation of Melamine-Induced Disruption in Testicular Oxidative Stress Pathways and Redox-Dependent Signaling in Adult Wistar Rats

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Abstract

Background: The growing presence of synthetic chemicals in the environment has caused serious concerns about their impact on the hormonal balance of humans and animals. Many of these substances, believed to disrupt the endocrine system, have been linked to reproductive problems worldwide. This includes rising infertility rates, particularly due to their negative effects on the health and function of the testes. ***Objective:*** This study was aimed at investigating the scavenging potentials of quercetin against melamine disruptive ability on the testes in animal models. ***Methodology:*** Thirty (30) adult male wistar rats were used for the study. They were randomly divided into six (6) groups of five (n=5) animals each. **Group A** rats were designated as negative control group and received 5ml/kg body weight of normal saline for 12 weeks. **Group B** rats received 300mg/kg melamine solution and 100mg/kg Quercetin for 12 weeks concomitantly. **Group C** rats received 600mg/kg melamine and 200 mg/kg quercetin for 12 weeks concomitantly. **Group D** rats received 300 mg/kg melamine and 100 mg/kg quercetin 6 weeks apart. **Group E** received 600mg/kg melamine and 200mg/kg quercetin, 6 weeks apart. **Group F** received 300mg/kg melamine as positive control. ***Result:*** Oxidative stress was induced by melamine in testicular tissues across the experimental groups leading to apoptosis of testicular cells seen in the distortion of testicular histomorphology and impaired hormonal function of the testes. ***Conclusion:*** Quercetin exhibited significant antioxidant activity, effectively mitigating oxidative damage induced by melamine exposure.

Keywords: melamine, quercetin, oxidative stress, endocrine disruption, testicular function

1. Introduction

Infertility and problems of impaired fecundity have been a concern through ages and is also a significant clinical problem today, which has been purported to affect between 15-20% of couples worldwide (Rowe, 2006; Saalu & Osinubi, 2009). Of the overall infertility cases, seeking clinical intervention, approximately 40-50% is due to "male factor" infertility and as many as 2% of all men are projected to exhibit suboptimal sperm parameters globally (Araoye, 2003; Abarikwu, 2013; Kumar & Singh, 2015; Sharma et al., 2021). It may be one or a combination of low sperm concentration, poor sperm motility, or abnormal morphology (Sharlip *et al*., 2002; PCASRM, 2015). The declining male fertility been observed could be traced and associated with congenital and acquired urogenital anomalies, infections of the genital tract, increased testicular temperature on account of varicocele, endocrine disturbances along with genetic distortions (WHO, 2000; Kumar & Singh, 2015). Endocrine disturbances are primarily made certain by Endocrine disrupting Substances (EDS), which can affect biological processes by several different modes of action. They may alter rate of synthesis or clearance of

endogenous hormones. They may also act as agonists or antagonists of normal hormones (Saalu & Osinubi, 2009). These substances may be synthetic in origin, such as; industrial chemicals, crop protection chemicals, or they may be natural like phytoestrogens and it has been documented that trace quantities of these chemicals are able to significantly trigger and sustain distortion of the endocrine system adversely impacting testicular function in males (Sharpe & Skakkebaek, 1993).

Endocrine disrupting chemicals (EDC) would likely exercise estrogenic and/or antiandrogenic effects, or directly trigger testicular toxicity by impairing Sertoli or Leydig cell function, increased oxidative stress, sperm DNA damage, or sperm epigenetic changes thereby adversely impacting overall testicular health and function (Sidorkiewicz et al., 2017). These chemicals have been described by WHO as a highly heterogeneous group of substances, including both manufactured chemicals and natural compounds, capable of altering the action of endogenous hormones (WHO, 2002). It is plausible that additional EDCs, which have recently increased in the environment, are contributing to current population declines in wildlife species, particularly those already challenged by other environmental stressors (Tubbs & McDonough, 2018; Marlatt et al., 2022). Within the scope of male reproductive biology, documented evidences have pointed to EDCs as teratogens; prompting hypospadias, cryptorchidism, abnormal sperm parameters, along with and overall distortion of the body's efficient physiology which is hitherto regulated and streamlined by various hormone signaling pathways (Wang & Baskin, 2008; Akunna et al., 2011; Akunna et al., 2013).

Melamine ($C_3H_6N_6$) is an organic compound with elevated nitrogen content, reported to have been synthesized about 190 years ago by Liebig (Zang et al., 2007) and it is conspicuously used in the production of durable plastic dishes, utensils, and kitchenware owing to its ability to withstand high temperatures (Bock & Ding, 1986; Zou & Chen, 2015). Melamine exposure subsists through several routes, including dietary sources, environmental contamination and occupational exposure with dietary sources of melamine contamination being the most pronounced (Kiekwe et al., 2024). Industrial discharge of melamine residues into the environment ensures environmental contamination, prompting melamine's contamination of water sources, soil and air (Wang et al., 2017; Wu et al., 2022) predisposing man and wild life to its impacting deleterious effects.

It is against this background that we sought to evaluate the antioxidizing potentials of Quercetin against the impact of melamine on testicular toxicity in adult Wistar rats, given the growing evidence of Melamine's endocrine disruptive activities adversely hampering overall testicular health and function (Zhang et al., 2012; Li et al., 2013; Chen et al., 2016; Gao et al., 2016; Yu et al., 2019; Wang et al., 2021).

2. Materials and Methods

In this study, thirty (30) adult male Wistar rats (13-16 weeks old) weighing 195-240g were procured from the animal house facility of the College of Health Sciences, Benue State University Makurdi and used for the study. The rats were randomly divided into six (6) groups (**A**-**F**) of five (5) rats each, were the average weight difference within and between groups was not in excess of ± 25% of the average weight of the sample population. **Group A** rats were designated as negative control group and received 5ml/kg body weight of normal saline for 12 weeks. **Group B** rats received 300mg/kg melamine solution and 100mg/kg Quercetin for 12 weeks concomitantly. **Group C** rats received 600mg/kg melamine and 200 mg/kg quercetin for 12 weeks concomitantly. **Group D** rats received 300 mg/kg melamine and 100 mg/kg quercetin 6 weeks apart. **Group E** received 600mg/kg melamine and 200mg/kg quercetin, 6 weeks apart. **Group F** received 300mg/kg melamine for 12 weeks as positive control. Administration of both melamine and quercetin solution was via the oral gavage. The rats were acclimatized for 14 days in plastic cages prior to commencement of the experiment, and were housed in plastic cages with adequate space to ensure easy movement under natural day and dark cycles (12hours light and 12 hours dark) at room temperature. The study was approved by the Research and Ethics Committee (REC) of the College of Health Sciences, Benue State University Makurdi in line with global guidelines for the use of laboratory animals.

Melamine (99%) by Sigma-Aldrich was procured from Bristol Scientific Company, Lagos State Nigeria. A stock solution was prepared in 1% carboxymethylcellulose (CMC). Quercetin was extracted from air dried and blended guava leaves via the Planar Chromatography method using N-butanol as extraction solvent as described by El Sohafy et al. (2009).

3. Animal Sacrifice

The animals were weighed and then sacrificed by cervical dislocation. The abdominal cavity was exposed via a midline incision to expose the reproductive organs and the testes were identified, excised and trimmed of all fat. Testicular weights of each rat were then taken using an electronic analytical and precision balance (BA 210S, d=0.0001 Sartorius GA, Goettingen-Germany). Post weighing, one of the testes was placed in a solution of Bouin's fluid and processed for histological light microscopic examination using routine hematoxylin and eosin, and immunohistological examination using Bax. The other testes were placed in a phosphate buffer of pH 7.2

with a dilution factor of 3mls per gram. Each of these tissues was separately transferred to a glass homogenizer containing the buffer solution and were homogenized using an electrical homogenizer (Remi 8000 RPM). The unbroken cells and cell debris were removed by centrifuging at 3000 RPM for 15 minutes. The obtained supernatants were used for the enzymatic estimations (antioxidant enzyme levels). Determination of follicle stimulating hormone (FSH), luteinizing hormones (LH), and testosterone were quantitatively measured by adopting an enzyme-linked immunosorbent assay (ELISA) technique using the Accu-Bind Microwell kit. Lake Forest, CA 92630, USA. Data from this study was expressed as mean ± standard deviation (SD) of number of experiments (n = 5). One-way ANOVA analysis was used to compare group means, while Turkey HSD post-hoc test was used to determine significant differences between group means, with mean differences considered statistically significant at $p<0.05$. Analysis of data was achieved with the Statistical Package for Social Sciences (IBM-SPSS)/ PC computer program (version 25.0 SPSS, USA edition).

4. Result and Discussion

The effect of the oral administration of quercetin on testicular weight of melamine-induced testicular toxicity is presented in Figure 1. The testicular weights were taken post sacrifice of the experimental animals.

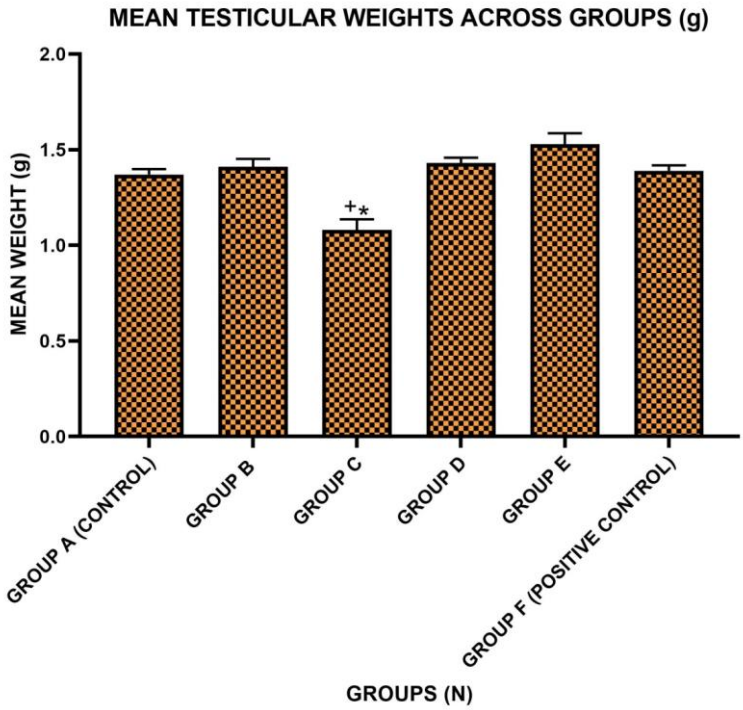


Figure 1. Simple Bar Chart Showing the Mean Testicular Weights across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

The result in Figure 1 shows a statistically significant decreases in testicular weight observed in Groups C compared to the control group. Group E was observed with the highest testicular weight gain. These findings point to the adverse impact of melamine on testicular weight observed in group C which was treated with 600mg/kg of melamine and 200mg quercetin concomitantly, whereas quercetin administered at 200mg/kg, six weeks apart in group E animals was able to influence a positive outcome of testicular weight, which had initially received 600mg/kg melamine in the first six weeks of treatment. Quercetin was previously reported to have positively impacted testicular weight resulting from exposure to other nano particles such as titanium dioxide -TiO_2 (Khorsandi et al., 2017).

5. Histomorphological Observations

The dimensions of the seminiferous tubules, including their diameter and height, were measured and compared across various experimental groups as shown in Figure 2. For the diameter, Group B-F all showed a statistically significant decrease in mean diameter of the seminiferous tubule when compared to the vehicle (CMC) and control groups respectively.

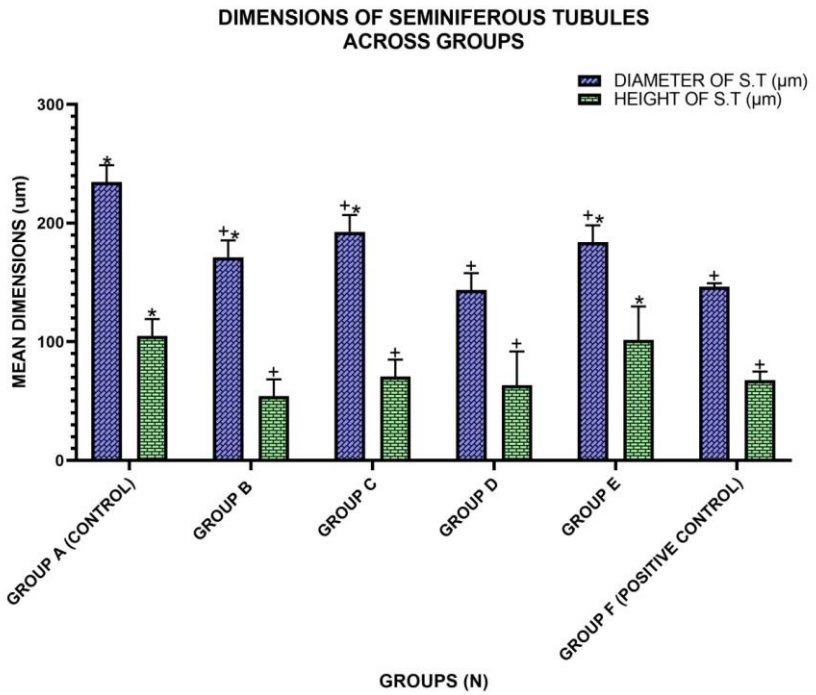
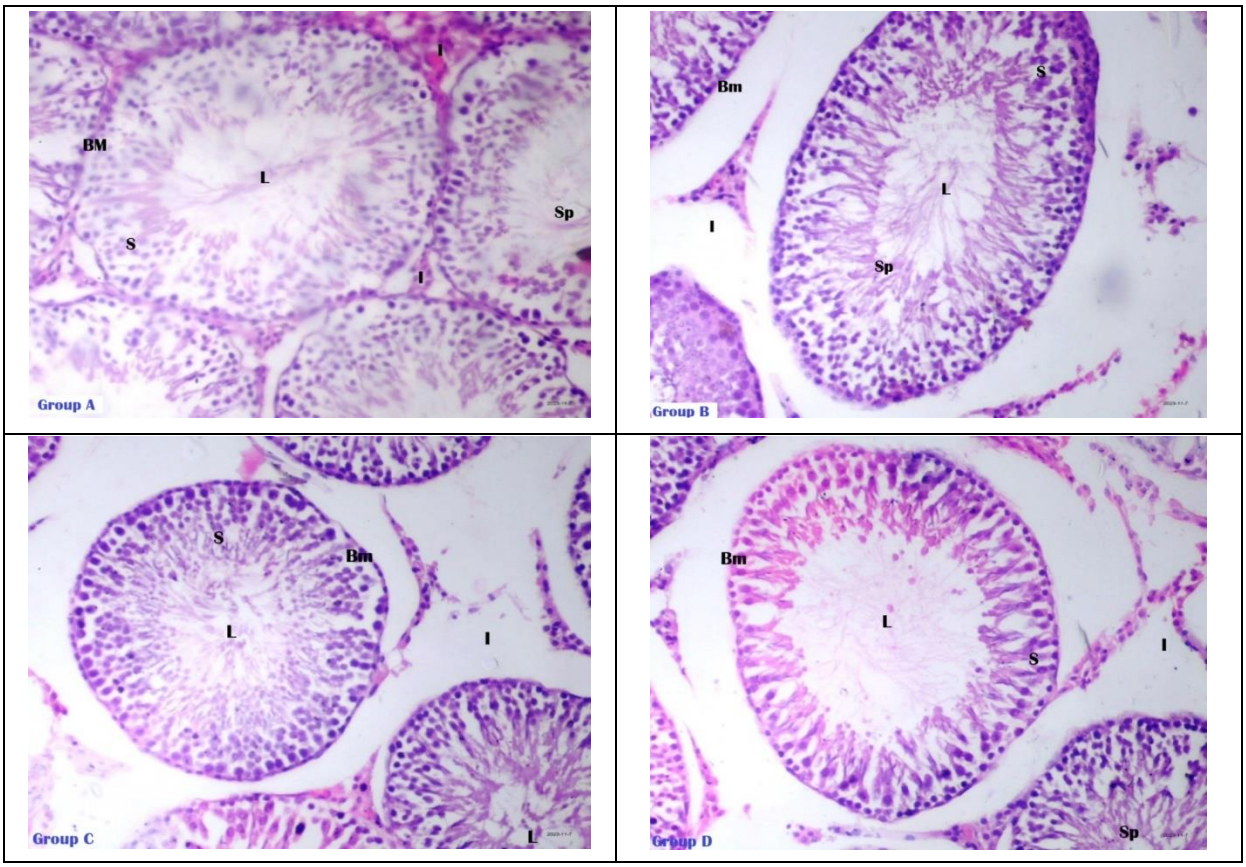


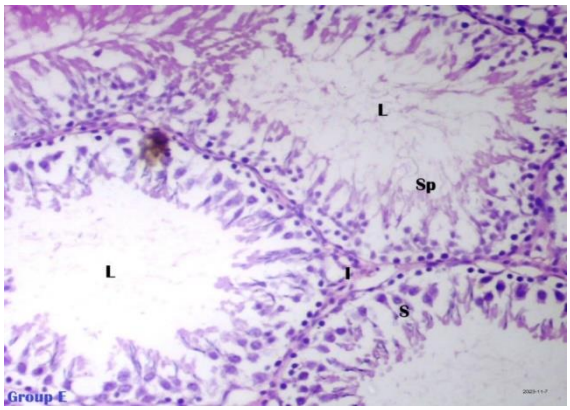
Figure 2. Simple Bar Chart Showing the Mean Dimensions of the Seminiferous Tubule across Groups

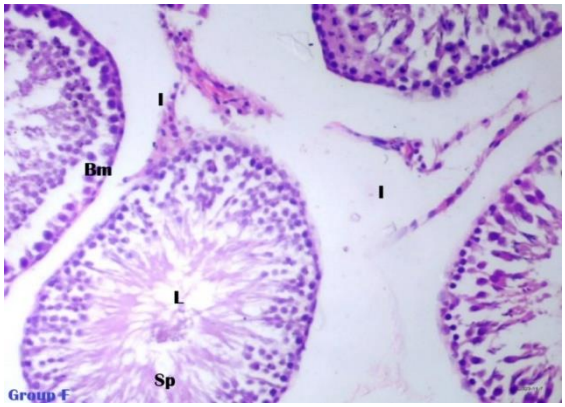
Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

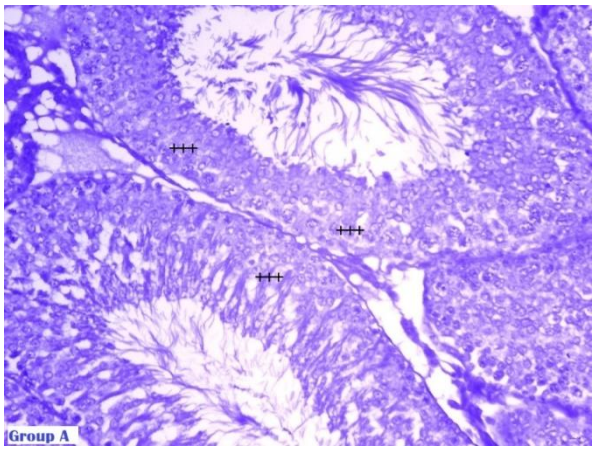
The result in Figure 2 indicates that the experimental groups B-F demonstrated significant reductions in both the diameter and height of the seminiferous tubules compared to the control group.

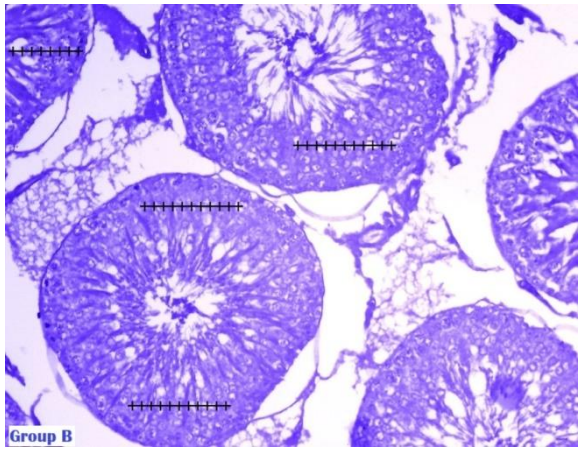


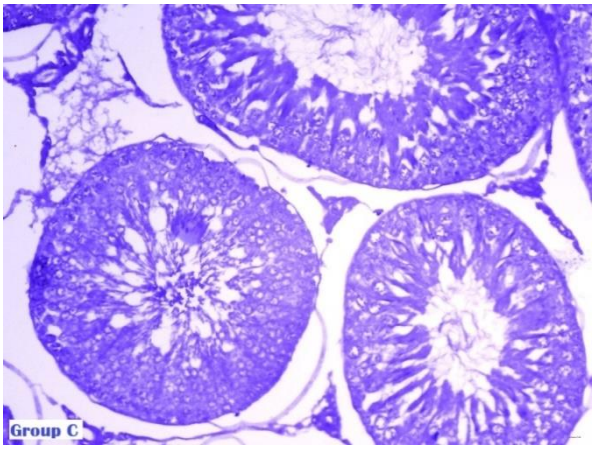


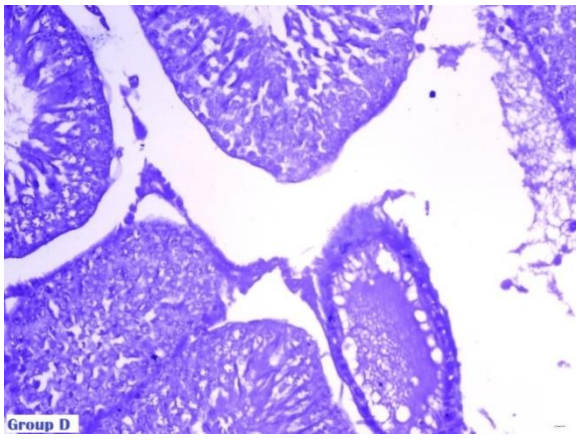


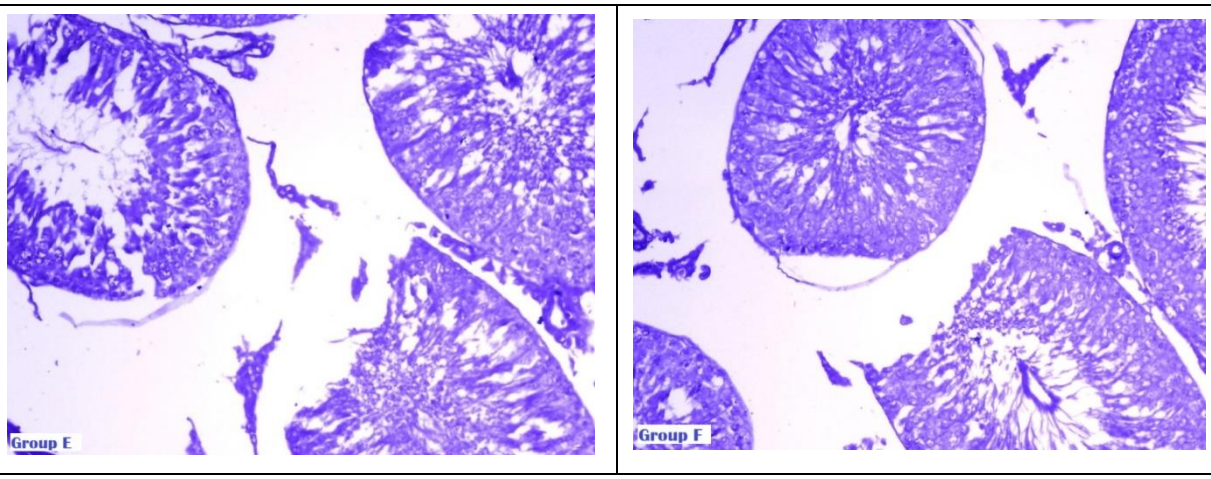
Histological analysis of testicular cross-sections from various experimental groups stained with H&E (× 400 magnifications) showed distinct differences in testicular histo-architecture. Group A (normal saline for 12 weeks) exhibited regular histo-architecture, while Groups B (300 mg/kg Melamine + 100 mg/kg Quercetin concomitantly for 12 weeks), C (600 mg/kg Melamine + 200 mg/kg Quercetin concomitantly for 12 weeks), E (600 mg/kg Melamine + 200 mg/kg Quercetin, 6 weeks apart), and F (300 mg/kg Melamine for 12 weeks) demonstrated varying degrees of distortion. Group D (300 mg/kg Melamine + 100 mg/kg Quercetin, 6 weeks apart) showed moderate distortion. Key features observed included the basement membrane (BM), interstitial space (I), lumen (L), and spermatozoa (SP).











Immunohistological analysis of testicular cross-sections stained for Bax (×400 magnification) revealed variations in cellular proliferation and tissue integrity across experimental groups. Group A (normal saline for 12 weeks) displayed regular cellular proliferation, while Groups B (300 mg/kg Melamine + 100 mg/kg Quercetin concomitantly for 12 weeks) and C (600 mg/kg Melamine + 200 mg/kg Quercetin concomitantly for 12 weeks) showed reduced cellular proliferation. Groups D (300 mg/kg Melamine + 100 mg/kg Quercetin, 6 weeks apart), E (600 mg/kg Melamine + 200 mg/kg Quercetin, 6 weeks apart), and F (300 mg/kg Melamine for 12 weeks) demonstrated progressively worsening testicular tissue distortion, with significant and severe distortion observed in Groups E and F, respectively. The degree of reactivity was marked using "+" signs.

6. Immunopositivity Reactions of the Testes

The immunopositivity reactions of the testes were measured using Bax marker across various groups. The mean levels were compared by one -way ANOVA analysis, as presented in Figure 3.

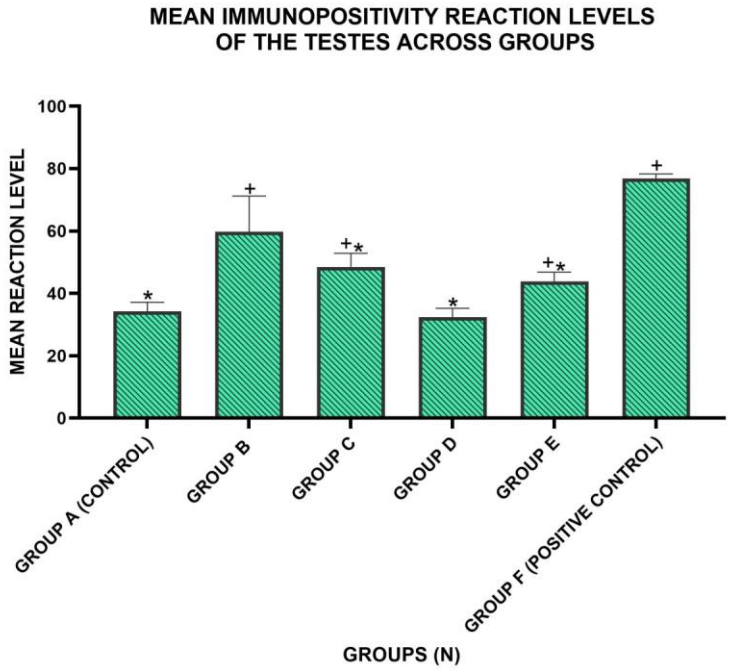


Figure 3. Simple Bar Chart Showing the Mean Immunopositivity Reaction Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

BAX Immunopositivity across groups B- E exhibited significantly elevated levels compared to the control group, pointing to increased apoptosis in these groups, whereas group F had the highest indication of apoptosis

owing to the highest immunopositivity reaction recorded. Groups D showed no significant difference in BAX levels compared to the control group, indicating that apoptosis levels were not markedly affected by the treatments in this group. This result suggests that the treatments administered to Groups B, C, E and F significantly affected apoptosis in the testes, as evidenced by the alterations in BAX immunopositivity levels. This result pointed to the histopathological changes seen in the testicular tissues across the treated groups, significantly affecting cell proliferation in the testes, as evidenced by the alterations in Bax immunopositivity levels, affirming previously reported opinions that the testes are particularly vulnerable to the toxic effects of melamine, which can lead to significant impairments in male fertility (Chen et al., 2009; Zhou et al., 2021), and testicular tissue atrophy (Zhao et al., 2015; Yang et al., 2016; Wu et al., 2019). Similarly, oxidative stress is here purported as melamine's primary mechanism of action via which its toxicity is observed on the testicular microenvironment. Previous findings reported melamine exposure to be associated with elevated levels of reactive oxygen species (ROS) leading to apoptosis (Guo et al., 2012) and reduced antioxidant defenses in testicular tissue, resulting in cellular damage and impaired sperm function (NTP, 2010; Shen & Wang, 2015; Lai & Sun, 2016) and that oxidative stress could induce lipid peroxidation of the sperm membrane, DNA fragmentation, and compromised sperm motility and viability (Chien & Chiang, 2014). The generation of free radicals (ROS) seen in this study by melamine, compromising sperm parameters for fertility has equally corroborated previous documentations on the incriminating role of ROS in idiopathic male infertility (Saalu et al., 2009b; Saalu, 2010). Quercetin is a lipophilic compound which is able to cross the cellular membranes and initiate multiple intracellular signaling pathways implicated in chemoprevention and one of quercetin's unique abilities is its dual function as a pro-oxidant or antioxidant (Watjen et al., 2005). An elevation in reactive oxygen species (ROS) results from oxidative stress and prompts DNA damage, promoting mutations. Mutations lead to uncontrolled growth of malignant tumor cells. Quercetin can reduce ROS by donating electrons and thereby decreased ROS-mediated DNA damage (Metodiewa et al., 1999; Boots & Haenen, 2008) consistent with the findings in groups B and C animals treated with quercetin and melamine concomitantly. This is the primary antioxidant mechanism of quercetin observed at its cellular concentrations which could be obtained by diet (Egert et al., 2008).

7. Anti-Inflammatory Markers: Interleukin 1, Interleukin 10 and TNF – α

Figure 4 presents the mean levels of anti-inflammatory markers (Interleukin 10, Interleukin 1, and Tumor Necrosis Factor-alpha) across different groups.

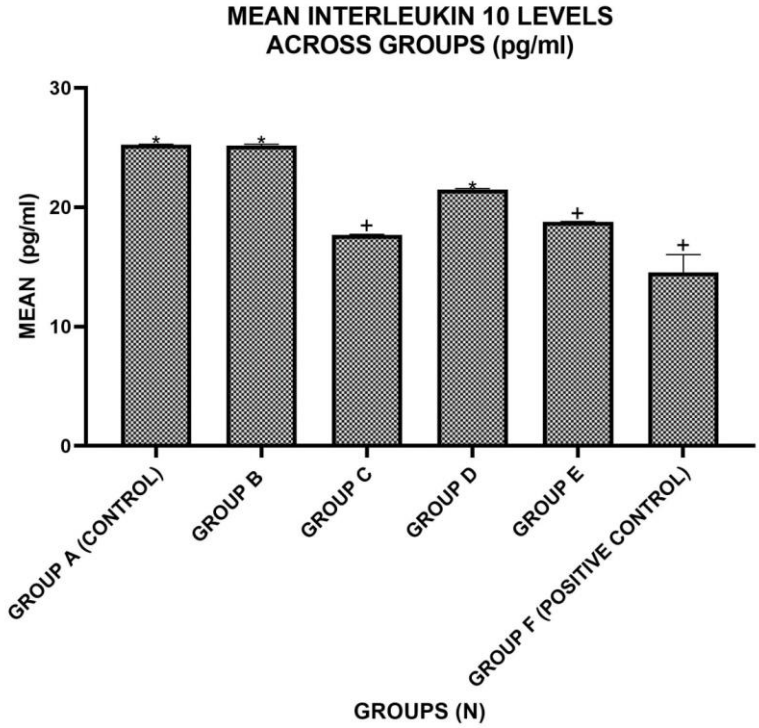


Figure 4. Simple Bar Chart Showing the Mean Interleukin — 10 Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

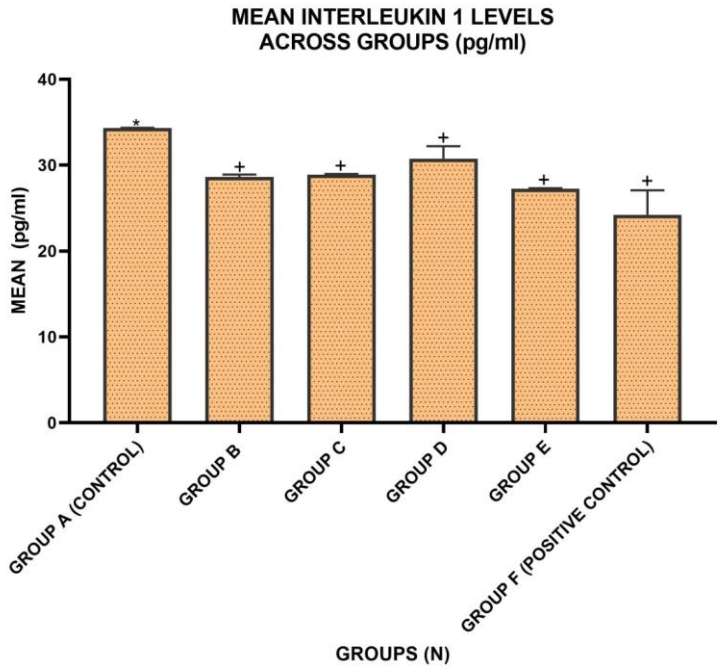


Figure 5. Simple Bar Chart Showing the Mean Interleukin — 1 Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

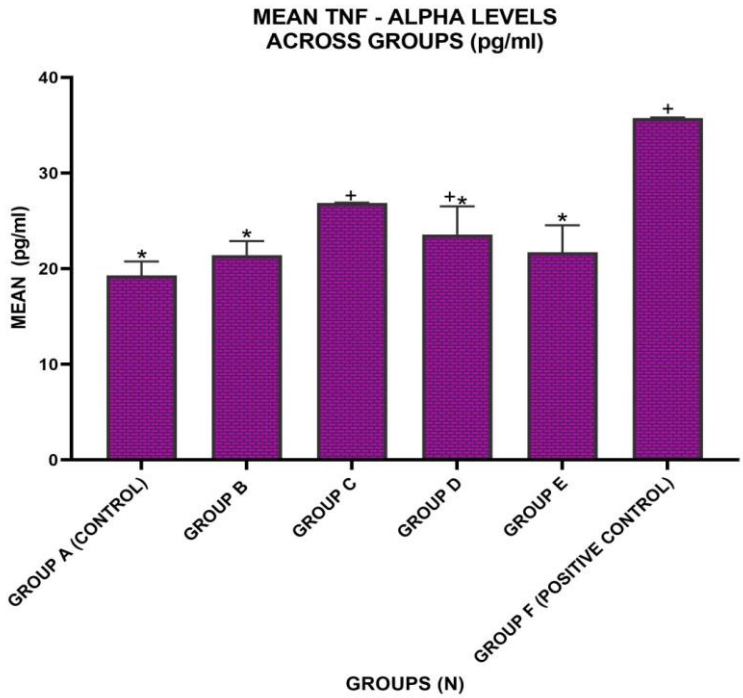


Figure 6. Simple Bar Chart Showing the Mean TNF — α Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

Interleukin 1 (IL-1): Groups B-F exhibited significantly lower IL-1 levels compared to the control group indicating a lower pro-inflammatory response.

Interleukin 10 (IL-10): Groups C-F had significantly lower IL-10 levels compared to Control group, suggesting a diminished anti-inflammatory response.

TNF-α: Groups C, D and F showed significantly higher TNF-α levels compared to Control group.

It was observed from this data that, for Interleukin 1 (IL-1); significantly lower levels in groups B-E suggest these groups experienced a reduced pro-inflammatory response, which could be beneficial in controlling inflammation.

For Interleukin 10 (IL-10); lower levels in groups C- E indicate a suppressed anti-inflammatory response in these groups. The significantly higher levels in group B suggest an enhanced anti-inflammatory effect.

For TNF-α; elevated levels in groups C and D indicate an increased inflammatory response. This contrasts with groups B and E where TNF-α levels did not significantly differ from control, implying these treatments did not exacerbate inflammation. These findings indicate varying efficacy of quercetin in modulating inflammatory responses, with some groups showing promising anti-inflammatory effects and others indicating potential pro-inflammatory responses. However, the marked elevation is observed in group F animals that received 300mg/kg melamine only, for 12 weeks. Pro-inflammatory cytokines like TNF-α in the testes could enhance certain physiological functions, but when they are higher than normal, they become deleterious to sperm (Azenabor et al., 2015). Comparably, El-Khadragy et al. (2020) reported an elevation in the proinflammatory cytokine TNF-α in the testes of rats after intraperitoneal lead exposure (20 mg/kg) for 7 days. Furthermore, the pro-inflammatory effects of NPs have been previously reported (Singh et al., 2007; Summer et al., 2024) and correlated with the binding of proteins to NP surfaces. For example, the adsorption of TNF-α on carbon black and aluminum oxide hydroxide (AlOOH) NP has been reported (Val et al., 2009). Additionally, exposure of rats to Al_2O_3NPs daily for 75 days resulted in a significant elevation of testicular levels of TNF-α (Yousef et al., 2019). As the inflammatory reactions within the cells are strongly connected with oxidative stress (Agarwal et al., 2018), the melamine-induced oxidative stress could be responsible for the increase in TNF–α immunoexpression. Contrastingly, Quercetin's coadministration and after destruction administration in this study suppressed TNF-α immune expression in the melamine + quercetin concomitant and curative treated groups, which reflects its anti-inflammatory effect (Saeeedi-Boroujeni & Mahmoudian-Sani, 2021). Against this background, the study of Nair et al. (2006) showed that a possible mechanism of quercetin-mediated suppression of TNF-α expression is mediated in downregulating NF-κβ1 gene expression.

8. Reproductive Hormones: Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), and Testosterone

Figure 7 shows the mean reproductive hormones levels across groups as compared by one – way ANOVA analysis.

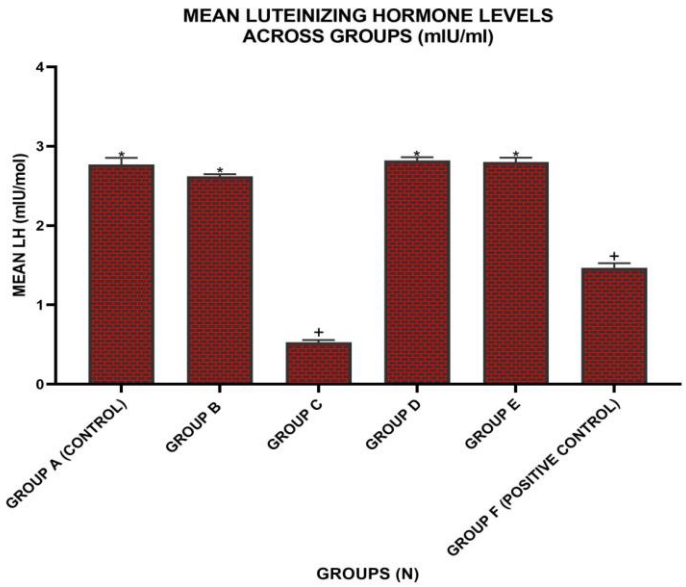


Figure 7. Simple Bar Chart Showing the Mean Luteinizing Hormone Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

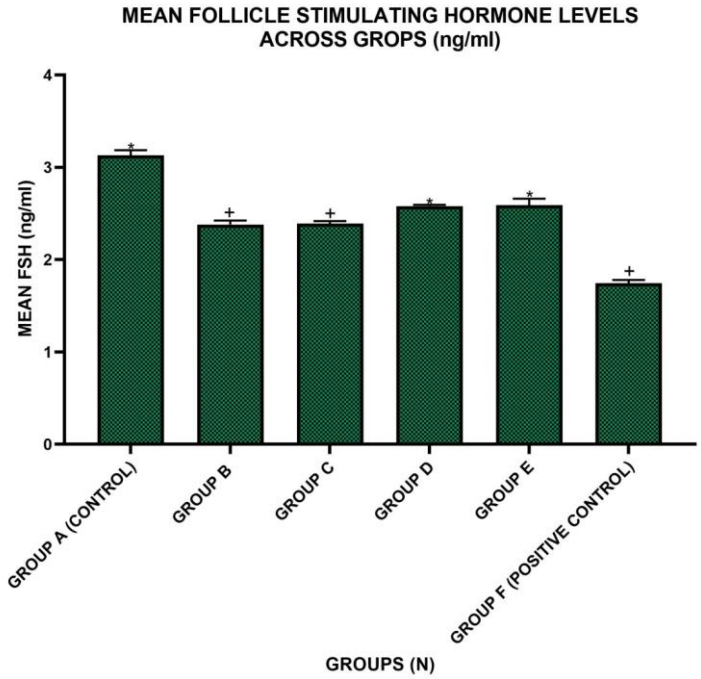


Figure 8. Simple Bar Chart Showing the Mean Follicle Stimulating Hormone Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

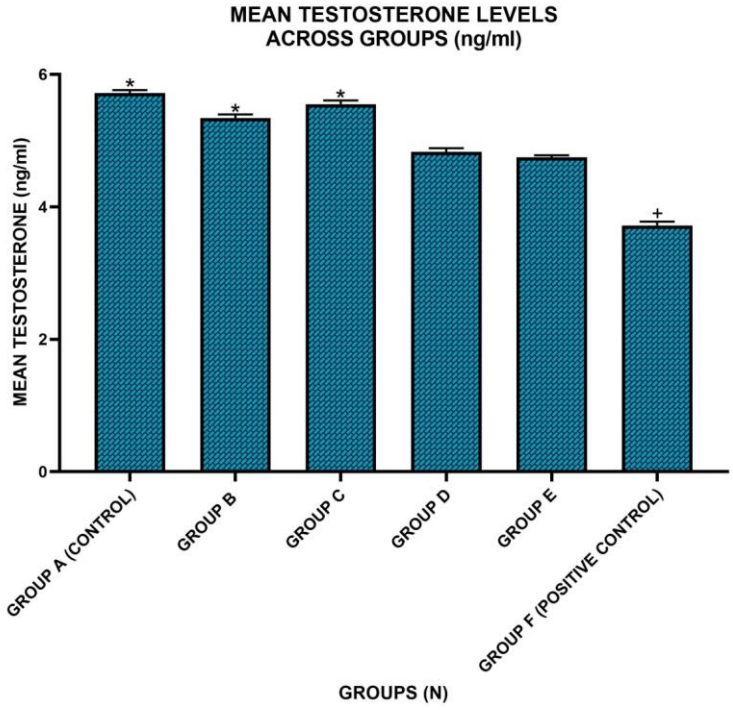


Figure 9. Simple Bar Chart Showing the Mean Testosterone Levels across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

The levels of reproductive hormones seen in this study points to the competing impact of melamine and quercetin treatments administered to Groups B-E, with observed optimal levels of reproductive hormone levels,

but marked suppressed level of LH in group C. Quercetin's resuscitative impact is observed on testicular function seen in testosterone levels been consistent across groups B-E compared with the control group. This result offers valuable insights into the hormonal dynamics across different experimental conditions, emphasizing the potential for melamine's disruptive ability in altering reproductive hormone levels, observed in group F, in relation to the combative effect of quercetin. Gonadotropins and testosterone are main regulators of germ cell development in the male and the development of a virile male germ cell is dependent on the right interplay of hypothalamus, pituitary and testes (Woode et al., 2012). Testosterone plays a key role in spermatogenesis and male fertility (Latif et al., 2008). The optimum serum levels of testosterone in groups B- E in this study points to a direct effect of quercetin, either on the leydig cell, or on the hypothalamic-pituitary axis (Sahreen et al., 2013). LH stimulates leydig cells to produce testosterone, while FSH binds with receptors in the sertoli cells to initiate spermatogenesis.

9. Sperm Count

Figure 10 shows the mean sperm count across the groups in this study, compared by one-way ANOVA.

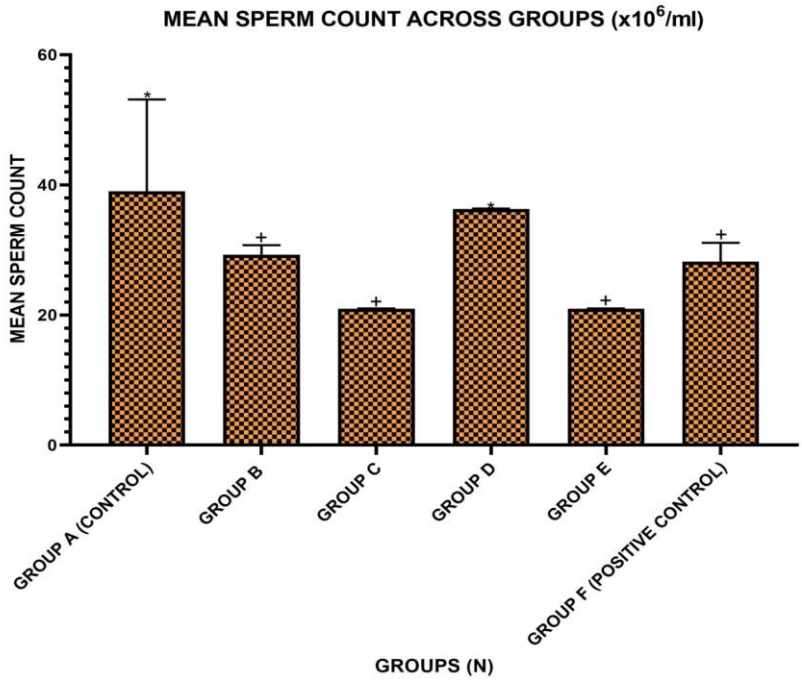


Figure 10. Simple Bar Chart Showing the Mean Sperm Count across Groups

Note: N = 5; + = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Control Group

* = Statistically Significant Difference in Mean at $p<0.05$ Compared to the Positive Control Group

The control group A had the highest mean sperm count, closely followed by group D. Group B also had a high mean sperm count, suggesting sustained positive effects from the intervention of quercetin on the disruptive effect of melamine. Groups C and E showed relatively low sperm counts, which points to the overwhelming disruption of testicular function my melamine in these groups. This result suggests that quercetin's treatment against melamine's disruptive impact on testicular function appear to have varied effects on sperm count across upon chronic exposure to melamine.

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