TIMED ADMINISTRATION OF FEBUXOSTAT IMPROVED TESTICULAR FUNCTION FOLLOWING TESTICULAR ISCHEMIA-REPERFUSION INJURY VIA INHIBITION OF MDA/NO PATHWAY, DOWNREGULATION OF TOLL-LIKE RECEPTOR 4 EXPRESSION AND RESTORATION OF REPRODUCTIVE HORMONES

Abstract

Introduction: Testicular ischemia-reperfusion injury (TIRI) generates reactive oxygen species (ROS) through xanthine oxidase (XO) activity in both ischemic and reperfusion phases leading to oxidative stress, inflammation, and disruption of reproductive hormones. Febuxostat (FEB), a xanthine oxidase (XO) inhibitor has been proven to exhibit superior antioxidant, anti-inflammatory, cytoprotective and anti-apoptotic effect than other XO inhibitors.

Methodology: Forty male Wistar rats (120-150 g) were divided into 5 groups (n=8 each): Sham operated (SO) rats underwent surgery without TIRI induction, torsion + detorsion (TD) rats underwent left unilateral TT for one hour and detorsed immediately to induce reperfusion which lasted for 3 days, Torsion + 5 mg/kg Febuxostat + Detorsion ($TF_{30}D$), Torsion + Detorsion + 5 mg/kg $Febuxostat_{imm}$ (TDF_{imm}) and Torsion + Detorsion + 5 mg/kg $Febuxostat_{30}$ (TFD_{30}). $TF_{30}D$, TDF_{imm} and TFD_{30} received 5 mg/kg of FEB intraperitoneally 30 minutes after TT onset, immediately on detorsion and 30 minutes after detorsion respectively. Rats were euthanized with 40 mg/kg ketamine 3 days after reperfusion. Blood samples were used for the measurement of nitrite, myeloperoxidase enzyme and reproductive hormones (LH, FSH, testosterone and inhibin). Left testes were homogenized and used for the assessment of Toll-like receptor-4-expression (TLR-4), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione (GSH), total protein (TP), non-protein thiol (NPSH) and protein thiol (PSH).

Results: This study showed that TIRI significantly increased oxidative stress markers (MDA, serum nitrite) and inflammatory markers (TLR-4, TNF-α, IL-1β) while reducing antioxidant enzymes (SOD, CAT, GSH, non-protein thiols) and altering reproductive hormones (increased LH, FSH, and decreased testosterone, inhibin) compared to SO group ($p<0.01$; 0.001).

Febuxostat administered during the ischemic phase (TF30D) led to the most significant reduction in oxidative stress markers, including MDA and serum nitrite, while also increasing total protein (TP) levels compared to the TD group ($p<0.001$). TF30D and TDFimm improved antioxidant defenses by increasing GSH and CAT levels, with TF30D specifically enhancing SOD and NPSH. Additionally, febuxostat significantly reduced inflammatory markers such as TLR-4, TNF-α, and IL-1β in all treatment groups, with MPO significantly lowered in TF30D and TDF30 ($p<0.001$;0.05). Hormonal balance (LH, FSH, inhibin, testosterone) was also restored in all febuxostat-treated groups ($p<0.01$;0.05; 0.001).

Conclusion: Febuxostat administered in the ischemic phase ($TF_{30}D$) is best to prevent TIRI when compared to its administration immediately on detorsion and 30 minutes after detorsion. This treatment strategy may guide clinician to prevent TIRI in humans after surgical detorsion.

Key words: Febuxostat; TIRI; time; inflammation; oxidative stress; antioxidant

enzymes; reproductive hormones.

1.0 INTRODUCTION

Testicular ischemia-reperfusion injury (TIRI) is the interruption of blood flow to the testes followed by its restoration (Eltzschig and Eckle, 2011). It frequently occurs after surgical detorsion (SD) of torsion of the testes, which has annual incidence rate of about one in 4,000 males (Sheth *et al.,* 2016). TIRI is a complication of surgical repair of twisted testes and its content. According to Ghasemnejad-Berenji *et al.* (2017), it may result in testicular damage and eventually infertility in male if not promptly and properly treated. Activation of oxidative stress pathway and toll-like receptor-4-induced inflammatory cascades as well as disruption of reproductive hormones may be involved in the etiology of TIRI-induced testicular damage (Molteni *et al.*, 2016; Almarzouq *et al.,* 2023).

The TIRI which is a two-phase process, involves the ischemic and reperfusion phases (Eltzschig and Eckle, 2011). The ischemic phase is characterized by production of xanthine oxidase (XO), a major reactive oxygen species (ROS) generator (Shafik *et al.,* 2013). In the ischemic phase of testicular torsion (TT), there is depletion of ATP which results in ROS production that interact with membrane lipid to cause lipid peroxidation which is harmful to testicular tissue (Kumar *et al.,* 2024). Lipid peroxidation in-turns alters the integrity and permeability of the testicular cells (Tuncer *et al.*, 2007). In addition to this, there is massive ROS production during reperfusion as a result of restoration of blood flow into the testes. This causes reperfusion injury characterized by interaction of ROS with nitric oxide to form hydroxyl radicals and peroxynitrite which are detrimental to testicular tissue (Ajike *et al.*, 2014; Marini *et al.,* 2012). By increasing lipid peroxidation and formation of hydroxyl and peroxynitrite radicals TIRI is capable of disrupting testicular function.

Furthermore, TIRI has been reported to trigger inflammation which plays a major role in testicular damage. Under physiological condition, sterile inflammation is a protective mechanism, however, during TIRI, the toll-like receptor 4 (TLR-4) expressed in the testes meant to maintain innate immunity becomes exaggerated and triggers massive inflammation. During the ischemic phase, the damaged associated membrane protein (DAMP) are activated which triggers TLR-4. This will results in the release of TNF-alpha and IL-1B from the macrophages into the blood. During reperfusion, these pro-inflammatory cytokines released into the blood will increase leukocyte recruitment (MPO) to the site of injury. This will increase intratesticular ROS and may inflict damage to the testes. In addition to this, previous studies have also reported that ROS generated during TIRI affects reproductive hormones via attack on Leydig cell and Sertoli cells of the testes (Al-Maghrebi *et al.,* 2016). Based on previous studies, xanthine oxidase (XO) generated during the ischemic phase is the originator of ROS production (Shafik, 2012; Yu *et al.,* 2023) which has to be blocked to prevent TIRI-induced infertility. Afolabi *et al.* (2022) also reported that XO-ROS activity is continued in the early phase of reperfusion and also contribute to ROS burst in the later phase (90-120 minutes) of reperfusion. For this reasons, there is a need to block ROS production in the ischemic phase, early phase of reperfusion and minutes after reperfusion to prevent TIRI induced testicular damage in the long-run. As a result of this, Ajike *et al.* (2024) administered febuxostat in the ischemic phase only, amlodipine on detorsion and vitamin E minutes after reperfusion. But the

effect of sole administration of febuxostat only either on detorsion or minutes after detorsion was not investigated. Hence, there is a need to investigate the effect of timed administration of febuxostat in the ischemic phase, immediately on detorsion and 30 minutes after detorsion to ascertain the time febuxostat administration would be most effective in preventing TIRI.

Febuxostat is a non-purine XO inhibitor with a favorable safety profile (Schumacher *et al.,* 2005). It has a superior effect in reducing ROS production compared to other XO inhibitors (Nomura *et al.,* 2014). Febuxostat is currently used in the hospital for the treatment of hyperuricemia (Bruce *et al.,* 2006) and gout but not in the management of TIRI. It has been reported to exhibit antioxidant (Rashad *et al.,* 2023), anti-inflammatory (Amirshahrokhi *et al.,* 2019), cytoprotective (Fahmi *et al.,* 2016) and anti-apoptotic properties (Krishnamurthy *et al., 2015).* It reno-protective effect against renal ischemia-reperfusion injury has previously been reported (Fahmi *et al.,* 2016; El-Shoura *et al.,* 2024). It has previously been reported to protect against myocardial ischemia-reperfusion injury (Wang *et al.,* 2015; Al-Kuraishy *et al.,* 2019). Afolabi *et al.* (2022) also reported that pretreatment with febuxostat protect against intestinal ischemia-reperfusion injury. Its protective role against ischemia-reperfusion injury in the skin flap of rats has also been documented (Odake *et al.,* 2021). This study therefore investigates the time febuxostat administration would be the most effective in preventing testicular damage after TIRI.

2.0 Materials and Methods

2.1 Experimental animal

Forty male Wistar rats, weighing 120- 150 g were purchased from the Animal House of Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Oyo State, Nigeria before the onset of the study. They were acclimatized for two weeks and kept throughout the experiment in well aerated plastic cages in the animal house (temperature 28-31^{0}C; photoperiod: 12-h natural light and 12-h dark; humidity:50-55%) of Faculty of Basic Medical Sciences (FBMS), LAUTECH, were fed with pelletized feed obtained from commercial dealer in Ogbomoso and watered *ad libitum*.

2.3 Drugs and reagents

All drugs and reagents used were of high analytical grade. Febuxostat and carboxymethylcellulose (CMC) solution were purchased from TCI chemicals, India (product number: FO840) and LOBA Chemie Pharmaceutical, Ltd. India: Product number: 0253000100.

2.4 Experimental Design

Forty (40) male Wistar rats were divided into 5 groups (n=8) rats as follows:

Group 1: The Control (Sham) rats underwent surgery, without TIRI induction received normal diet and distilled water

Group 2: Torsion + Detorsion (TD) rats underwent left unilateral testicular torsion (TT) for one hour and testicular detorsion followed which lasted for 3 days.

Group 3: Torsion + Febuxostat + Detorsion ($TF_{30}D$) rats received 5 mg/kg of febuxostat intraperitoneally (i.p) after 30 minutes of TT and testicular detorsion followed 30 minutes later, which lasted for 3 days.

Group 4: Torsion + Detorsion + $Febuxostat_{imm}$ (TDF_{imm}) rats received 5 mg/kg of febuxostat

(i.p) immediately on detorsion. That is, 60 minutes after TT.

Group 5: Torsion + Detorsion + Febuxostat$_{30}$ (TFD$_{30}$) rats received 5 mg/kg of febuxostat (i.p) after 30 minutes of detorsion.

The rats were administered with febuxostat 30 minutes after testicular torsion induction, immediately on detorsion and 30 minutes after detorsion intraperitoneally once throughout the experiment. Selected dosage of febuxostat was according to Wang *et al.* (2015) and Ajike *et al.* (2024).

2.5 Experimental induction of testicular ischemia-reperfusion injury

The rats were fasted for 12 hours before the experiment. They were weighed and anaesthetized with Ketamine (50 mg/kg) and Xylazine (10 mg/kg) intraperitoneally (Herrmann *et al.,* 2019).The rats were restrained on the dissecting board. The left scrotal, perineal and inguinal areas of the rats were shaved and cleaned with methylated spirit. The left testis was firmly grasped and the caudal epididymis was located and used as a reference point. A high left scrotal incision was made to slightly open up the tunica vaginalis to locate the testis. The edges of the tunica vaginalis was clamped with toothed dissecting forceps to produce a tissue plain. The essence of this is to enhance easy returning of the testes back into the scrotum. A gentle pressure was applied to push the left testes out. The gubernaculum testes was located and cut off to free the left testes. The freed left testis was twisted at 720^0 in a clockwise direction to induce ischemia for one hour. A pouch was created in the scrotum with a long surgical scissors into which an anchoring suture was passed from outside into the inside and attached to the tuft of tissue in-between the testes and epididymis and then passed outward and pulled down to ensure the testes is returned into the scrotum to remain in a twisted state. The incision site was closed up with 2-0 chromic suture. After one hour of torsion, the rats were opened up to untwist the testes to induce reperfusion which lasted for 3 days. This procedure was according to the method of Afolabi *et al.* (2022).

2.6 Animal sacrifice, blood and serum collection

Three (3) days after reperfusion, the rats were anaesthetized with ketamine (50 mg/kg). Blood was collected through retro-orbital puncture using heparinized capillary tube and introduced into the plain bottles. The blood collected into the plain bottles were allowed to clot for 15 minutes and then centrifuged at 2500 revolutions per minutes for 15 minutes to obtain serum. The serum was collected into Eppendorf bottles with Pasteur pipettes and refrigerated for further assays.

2.7 Tissue collection and preparation of testicular homogenate

Testicular tissue were harvested and cleared of adherent tissue. They were weighed, homogenized and centrifuged for assay of biochemical parameters.

2.8 Assessment of Testicular weights

Testicular weight were measured with sensitive weighing scale (Lisay, China).

2.9 Biochemical Analysis

Testicular homogenates was used to assess superoxide dismutase (SOD) activity spectrophotometrically using the protocol of Paoletti *et al,* (1986). Catalase activity was assessed spectrophotometrically at 570-610 nm using the method of Anjum, (2016), MDA concentration was evaluated according to the method of Adegunlola *et al.* (2012). Glutathione (GSH) concentration, protein and non-protein thiol were assessed by the method of Williams, (2014). Serum nitrite concentration was assessed by checking the nitrite level as described by Tatsch *et al.* (2011).

2.10 Inflammatory Markers

Toll-like receptor-4 expression was measured in testicular supernatants using ELISA kits in accordance with the manufacturer's instructions. Myeloperoxidase (MPO) activity was assess as described by Pulli *et al.* (2013). TNF-α and IL-1β were measured in serum.

2.11 Reproductive Hormones

Serum FSH, LH, inhibin and testosterone hormone concentration were measured from blood samples taken from the retro-orbital and centrifuged at 2,500 per min to obtain serum. Total serum concentrations of FSH, LH and Testosterone were measured using ELISA kit. LH (CALBIOTECH catalog No LH232F 96 test), FSH (CALBIOTECH catalog No FSH232F 96 tests), testosterone (Bio-Inteco Catalog No 10007) and Inhibin B (Elabscience E-EL-H010).

2.12 Statistical analysis

Data were expressed as mean ± standard error of mean (Mean ± SEM). Analysis was performed with Graph Pad Prism, Version 7.0 (Graph Pad software, Inc., USA) was used to compare within group and Tukey's Post-test was used for multiple comparison P-Values less than 0.05 were considered statistically significant.

3.0 Results

The obtained testicular biochemical parameters such as SOD, CAT, GSH, non-protein thiol, and total protein were significantly decreased during torsion + detorsion (reperfusion injury), while MDA, protein thiol, and serum nitrite were significantly increased during torsion + detorsion. Tissue SOD ($p<0.05$), CAT ($p<0.05$), GSH ($p<0.05$), non-protein thiol ($p<0.05$), and total protein ($p<0.05$) were significantly decreased in torsion + detorsion group rats when compared with rats in the sham group, while tissue MDA ($p<0.05$), protein thiol ($p<0.05$), and serum nirite ($p<0.05$) were significantly increased in torsion + detorsion group rats when compared to the sham group. Tissue SOD was significantly increased ($p<0.05$) in $TF_{30}D$ and TDF_{imm} only, CAT was significantly increased in $TF_{30}D$ only ($p<0.05$), GSH was significantly increased in $TF_{30}D$ only ($p<0.05$), non-protein thiol was significantly increased in $TF_{30}D$ and TDF_{imm} only ($p<0.05$), and total protein was significantly increased in all the treated groups ($TF_{30}D$, TDF_{imm}, and TDF_{30}) ($P<0.05$), while testicular MDA, protein thiol, and serum nitrite were significantly decreased in all the groups as well ($TF_{30}D$, TDF_{imm}, and TDF_{30}) (febuxostat $P<0.05$) (Fig. 1A, B, C, D, E, F, G, and H).

IL-1β, TLR-4, TNF-α, and MPO were increased ($p<0.01$) in the Torsion + Detorsion group when compared to the sham group, but all the treated groups significantly reduced IL-1β ($TF_{30}D$, TDF_{imm}, and TDF_{30}) ($p<0.05$), TLR-4 was significantly decreased in $TF_{30}D$ only ($p<0.05$; 0.01), and TNF-α and MPO were significantly increased in $TF_{30}D$ and TDF30only ($p<0.05$;0.01; 0.001) (Fig. 2A, B, C, and D).

LH and FSH were increased ($p<0.01$; 0.001) in the Torsion + Detorsion group when compared to sham group, while inhibin and testosterone were decreased ($p<0.05$; 0.01) in the Torsion + Detorsion group when compared to sham group. In all the treated groups, LH and FSH significantly reduced ($TF_{30}D$, TDF_{imm}, and TDF_{30}) ($p<0.05$; 0.01; 0.001), while inhibin and testosterone significantly increased in all the treated groups as well ($TF_{30}D$, TDF_{imm}, and TDF_{30}) ($p<0.05$; 0.01; 0.001) (Fig. 3A, B, C, and D).

4.0 Discussion

This study investigates the effect of febuxostat on testicular function when administered 30 minutes after testicular torsion (TT), immediately on detorsion and 30 minutes after detorsion. The essence of this is to establish the time at which febuxostat administration will be most effective. Testicular torsion and its repair may cause male infertility via depletion of antioxidant enzymes which results into massive ROS production that can up-regulate toll-like receptor to exacerbate inflammatory response and inflict injury to the Leydig and Sertoli cells. In this study, antioxidant markers such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), total protein (TP) and non-protein thiol (NPSH) were investigated. This markers help to protect the integrity of the testes against ROS generated during testicular ischemia-reperfusion injury (TIRI). For instance, SOD is an antioxidant which serves as the first line of defense against oxidative stress. It converts superoxide radicals (O^{-2}) into oxygen (O_2) and hydrogen peroxides (H_2O_2) (Ighodaro *et al.,* 2018) while catalase on the other hand break down toxic hydrogen peroxide into water and oxygen. Glutathione (GSH) functions as a free radical scavenger and a substrate for various enzymatic reactions involved in detoxification and redox regulation (Couto *et al.,* 2016). Non-protein thiol contain sulfhyldryl (SH) group which help to scavenge free radicals while total protein helps to maintain the integrity of the protein component in the testes.

In this study, the observed depletion in the antioxidant defense system (SOD, catalase, GSH, and thiol activities) and elevation of oxidative stress makers (MDA and Serum NO) following TIRI in TD group is an indication of oxidative stress compared to SO (Tsaturyan *et al.,* 2022). Antioxidant enzymes are essential for preserving the body's redox balance. Superoxide Dismutase (SOD) is an antioxidant enzyme that produces oxygen (O_2) and hydrogen peroxides (H_2O_2) by dismutating superoxide radicals (O^{-2}), (Ighodaro *et al.,* 2018); GSH functions as a free radical scavenger and a substrate for various enzymatic reactions involved in detoxification and redox regulation (Couto *et al.,* 2016); and the antioxidant activity of catalase is dependent on the degree of SOD activity because it breaks down hydrogen peroxide (H_2O_2) into water and oxygen (Ighodaro *et al.,* 2018). Thiols have an antioxidant effect when the thiol (cysteine) residue oxidizes and forms a disulfide (GSSG), which glutathione reductase then reduces back to the thiol form (GSH) (Kukurt *et al.,* 2021). It has been proposed that oxidative damage and decreased glutathione levels are early, potentially signaling processes in apoptotic cell death (Circu *et al.,* 2012). Treatment with febuxostat at $TF_{30}D$ showed a superior effect in preventing oxidative stress than TDF_{imm} and TDF_{30} by increasing the activities of SOD and catalase in the testicular tissue (Douzinas *et al.,* 2019).

Febuxostat, which is a potent XO inhibitor, could have exerted its protective effects by preventing ROS generation thereby alleviating the burden on these antioxidant enzymes and

preserving their activities (Wang *et al.,* 2015; Kim *et al.,* 2020). Thiols (GSH and non-protein thiol) depletion in TD group indicates their utilization under oxidative stress to neutralize ROS and absence of thiol (cysteine) residue following torsion/detorsion (Georgescu *et al.,* 2022). Administration of FEB at TDF_{imm} and TDF_{30} validates that FEB maintains endogenous thiol concentration following TD via XO-induced oxidative stress (Xu *et al.,* 2008). Interestingly, the findings from this study prove that administration of FEB at TDF_{30} is not a feasible treatment regime to prevent non-protein thiol depletion and oxidative stress. Treatments with FEB at $TF_{30}D$, TDF_{imm}, and TDF_{30} effectively suppressed XO-induced free radical generation with consequent MDA generation, thereby mitigating lipid peroxidation (Kim *et al.,* 2020). The surge in oxygen radicals post-detorsion triggers eNOS activation in vascular endothelium, promoting L-arginine oxidation and subsequent NO production (Wijaya *et al.,* 2022). The interaction between NO and free radicals yields more reactive and hazardous nitrogen species (Galiniak *et al.,* 2023). The reduction in NO bioavailability mediated by XO can be a significant source of reactive nitrogen species, such as peroxynitrites. This study demonstrates that FEB administration at $TF_{30}D$, TDF_{imm}, and TDF_{30} inhibit lipid peroxidation reactions, eNOS activation, and NO bioavailability, exerting its effects at the mitochondrial level by suppressing XO-induced free radical generation (Kim *et al.,* 2020).

Inflammation is a complex biological response triggered by the body's immune system in response to various harmful stimuli, such as pathogens or damaged cells. While inflammation is a vital defense mechanism, excessive or dysregulated inflammation can contribute to the pathogenesis of various diseases, including autoimmune disorders, cardiovascular diseases, neurodegenerative diseases, cancer, and ischemia reperfusion injury (Chen *et al.,* 2018; Furman *et al.,* 2019). In this study, it was observed that TLR-4 expression was increased in the TD when compared with the SO. Its upregulation in the TD suggests that testicular torsion and subsequent reperfusion trigger an inflammatory response, which is a well-documented phenomenon in IRI (Kalogeris *et al.,* 2014). The TLR-4 is a pattern recognition receptor which initiates the innate immune response and inflammatory cascade (Zhu and Mohan, 2010). TLR4 activation triggers downstream signaling cascades, such as the myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways, leading to the production of pro-inflammatory cytokines and chemokines (Lu *et al.,* 2008). The administration of febuxostat, a xanthine oxidase inhibitor, at different time points ($TF_{30}D$, TFD_{imm}, and TFD_{30}) effectively attenuated the increase in TLR4 expression compared to the TD. Also, higher TLR4 levels was observed in the TFD_{imm} and TFD_{30} compared to the $TF_{30}D$ indicating that administering febuxostat immediately or 30 minutes after detorsion may be less effective in mitigating the inflammatory response compared to its administration 30 minutes after torsion. Febuxostat administered 30 minutes after torsion ($TF_{30}D$) may have effectively reduced ROS production during the early stages of reperfusion, thereby attenuating the downstream inflammatory cascade and TLR-4 upregulation. Tumor necrosis factor-alpha (TNF-α) concentration was significantly increased in the TD when compared to the SO. TNF-α is a pro-inflammatory cytokine which plays a central role in initiating and amplifying the inflammatory cascade, leading to tissue damage and dysfunction (Patel *et al.,* 2015). Oxidative stress and ROS generation during IRI can lead to mitochondrial dysfunction, triggering apoptotic pathways and the release of DAMPs (Kalogeris *et al.,* 2014). These DAMPs can activate inflammatory signaling cascades and induce the production of pro-inflammatory

cytokines like TNF-α. In contrast, there was a significant decrease in TNF-α levels in the $TF_{30}D$, TFD_{imm}, and TFD_{30} compared to the TD. This finding is consistent with previous studies which have demonstrated the anti-inflammatory properties of febuxostat in various pathological conditions (Amirshahrokhi, 2019; Mizuno *et al.,* 2019). Febuxostat can modulate various inflammatory signaling pathways, such as NF-κB, MAPK, and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways (Mizuno *et al.,* 2019). Its inhibition of these pathways can attenuate the production of pro-inflammatory cytokines, including TNF-α, thereby reducing inflammation and tissue injury. Notably, this study further showed that the timing of febuxostat administration appears to influence its efficacy in mitigating TNF-α production. There was an observed increase in TNF-α levels in the TFD_{imm} and TFD_{30} compared to the $TF_{30}D$, suggesting that administering febuxostat 30 minutes after torsion ($TF_{30}D$) is more effective in reducing TNF-α production than administering it immediately or 30 minutes after detorsion.

IL-1β, like TNF-α, is a pro-inflammatory cytokine that plays a crucial role in initiating and amplifying the inflammatory cascade, leading to tissue damage and dysfunction (Dinarello, 2018). The observed increase in IL-1β concentration in the TD compared to the SO attests to the inflammatory response triggered by TIRI. Febuxostat administered at the ischemic phase ($TF_{30}D$) and reperfusion phase (TFD_{imm}, and TFD_{30}) showed a significant reduction in IL-1β levels compared to the TD but the reduction was more in $TF_{30}D$ compared to TFD_{imm}, and TFD_{30}. The observed results suggest that febuxostat effectively mitigates the production of IL-1β in testicular IRI, likely through modulation of various interrelated pathways including the TLR signaling pathway, ROS-activated NLRP3 inflammasome pathway, and MAPK pathways, among others. The MAPK pathway include the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK cascades, which are involved in the regulation of inflammatory gene expression, including IL-1β (Amirshahrokhi, 2019; Mizuno *et al.,* 2019). Unlike TLR-4 and TNF-α, TF30D did not significantly reduce IL-1β level compared to the other treated. This reveals that febuxostat, given 30 minutes after torsion, immediately on detorsion, and 30 minutes after detorsion, had similar effects on IL-1β levels. The lack of oxygen and nutrient supply during ischemia leads to the accumulation of hypoxanthine and xanthine, which culminates in the generation of ROS (Wu *et al.,* 2018). ROS activates various inflammatory signaling pathways, including the NLR family pyrin domain containing 3 (NLRP3) inflammasome, which is a key regulator of IL-1β production (Tschopp and Schroder, 2010).In concurrence with previous studies, increased myeloperoxidase (MPO) activity in the TD compared to the SO demonstrate the involvement of neutrophils in the pathogenesis of TIRI and their contribution to tissue damage (Kalogeris *et al.,* 2014; Wu *et al.,* 2018). MPO is an enzyme primarily found in neutrophils and is widely used as a marker of neutrophil accumulation and activation (Güler *et al.,* 2022). ROS produced during TIRI can act as potent chemo-attractants for neutrophils and contribute to their activation and degranulation, leading to the release of MPO and other inflammatory mediators (Güler *et al.,* 2022). Among the treated, only the $TF_{30}D$ showed a significant decrease in MPO concentration when compared to the TD, further buttressing the point that febuxostat given during the ischemic phase can help mitigate the inflammatory response. This finding implies that febuxostat exerts a protective effect against IRI-induced inflammation, likely through its ability to reduce oxidative stress via modulation of the xanthine oxidase pathway.

Reproductive hormones including testosterone, FSH, LH, and inhibin are markers of male sexual health, they are essential for sperm production, sexual development, and overall function. Spermatogenesis, the process of sperm formation, is tightly regulated by testosterone and gonadotropins (Ramaswamy *et al.,* 2014; Anso *et al.,* 2023). Studies show that TIRI impairs testicular function, decreasing inhibin and testosterone levels, while increasing luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels. Consistent with these findings, this study observed elevated LH and FSH levels, alongside reduced testosterone and inhibin. Inhibin, produced by the testes, helps regulate testosterone production. TIRI disrupts this process by diminishing inhibin production (Kongmanas *et al.,* 2008). Testosterone and inhibin synthesis in the testes are controlled by the hypothalamus and pituitary gland through the release of FSH and LH, maintaining hormonal balance via a negative feedback loop (McQuaid *et al.,* 2014). Elevated levels of LH and FSH, along with decreased inhibin and testosterone, suggest that TIRI-induced oxidative stress has damaged Leydig and Sertoli cells, impairing testicular function. Previous research has also demonstrated TIRI's adverse effects on Leydig and Sertoli cell activity (Al-Maghrebi *et al.,* 2016). Consistent with other studies, the observed rise in LH and decline in testosterone levels compared to the sham-operated (SO) group suggests the potential onset of primary hypogonadism due to blood flow restriction and subsequent reperfusion injury (Traish *et al.,* 2015). Additionally, the observed increase in FSH, a key hormone in spermatogenesis, highlights reduced sperm production (Chen *et al.,* 2017). Testicular ischemia-reperfusion injury (TIRI) has been shown to cause a decrease in testosterone and inhibin levels, as reported by Kongmanas *et al.* (2008). Inhibin is a hormone produced by the testes that plays a crucial role in regulating testosterone production. TIRI disrupts the normal function of the testes by reducing inhibin production, leading to imbalances in hormone levels. Testosterone and inhibin synthesis in the testes are regulated by the hypothalamus and pituitary gland, which control the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This process is maintained by a negative feedback loop that ensures balance between testosterone and inhibin levels (McQuaid *et al.,* 2014).

The elevated LH and FSH levels observed in testicular dysfunction (TD) alongside the reduced levels of testosterone and inhibin suggest that reactive oxygen species (ROS) generated during TIRI may have caused damage to Leydig and Sertoli cells, which are essential for testicular function. This disruption impairs the production of testosterone and inhibin, key hormones in male reproductive health. Al-Maghrebi *et al.* (2016) also documented that TIRI negatively impacts the activity of Leydig and Sertoli cells, leading to diminished hormonal production. Similar to other studies, the observed rise in LH and decrease in testosterone in TD compared to sham-operated (SO) groups raises the possibility of primary hypogonadism, a condition where impaired testicular function leads to reduced hormone production. This may result from restricted blood flow and subsequent tissue repair, as noted by Traish *et al.* (2015). Furthermore, the elevated FSH levels seen in TD highlight the disruption in spermatogenesis, as FSH plays a critical role in sperm production (Chen *et al.,* 2017). The overall hormonal imbalance caused by TIRI suggests significant damage to the testicular cells, leading to impaired reproductive function. Febuxostat has been shown to raise testosterone levels in a study by Damiani *et al.* (2023) when compared to testicular dysfunction (TD), by stimulating the activity of epidermal growth factor in Leydig cells, which are essential for cell survival. This effect was observed when febuxostat was administered during both the ischemic phase (TF30D) and the reperfusion phase (TDFimm and TDF30).

Increased testosterone levels were seen in TF30D and TDFimm when compared to TDF30, which contributed to the reduction in luteinizing hormone (LH) during the ischemic and reperfusion phases. This reduction in LH production can be attributed to the negative feedback mechanism that regulates hormone levels, whereby elevated testosterone inhibits the hypothalamus and pituitary gland from producing more LH (Pitteloud *et al.,* 2008). Testosterone and follicle-stimulating hormone (FSH) work in tandem to stimulate spermatogenesis within the testes. In this study, the reduction in FSH levels observed in the TF30D, TDFimm, and TDF30 groups suggests a restoration of hormonal balance and improved spermatogenesis (Santi *et al.,* 2020). Sertoli cells play a critical role in spermatogenesis by secreting inhibin, which regulates this process. Inhibin enhances the mitotic activity of spermatogonia and provides structural and nutritional support to germ cells (Griswold *et al.,* 2018). The increased inhibin levels in $TF_{30}D$, TDFimm, and TDF_{30} indicate improved Sertoli cell activity and enhanced spermatogenesis. Additionally, inhibin suppresses FSH production via its action on the pituitary gland (Makanji *et al.,* 2011). The administration of febuxostat during the ischemic ($TF_{30}D$) and reperfusion (TDFimm, TDF30) phases appears to block the buildup of reactive oxygen species (ROS), thereby improving hormone levels and supporting testicular function.

CONCLUSION AND RECOMMENDATION

Treatment with febuxostat at $TF_{30}D$ has shown promising effects in improving antioxidants, oxidative stress and inflammatory markers compared to TDFimm and TDF30. Also, administration of febuxostat at $TF_{30}D$ alleviated hormonal and testicular damage caused by testicular ischemia-reperfusion injury (TIRI) to enhance spermatogenesis and Sertoli cell function. Febuxostat administration during ischemic phase ($TF_{30}D$) should be explored as a therapeutic strategy to protect testicular function against xanthine oxidase-driven ROS production in cases of TIRI. Further studies are recommended to optimize the dosing of febuxostat administration to maximize its protective effects.

2.2 Ethical Approval

Ethical approval was obtained from Faculty of Basic Medical Sciences, LAUTECH Ogbomoso with the reference number: 027/05/2024

The animal handling procedure was carried out in accordance with guidelines for the use and care of laboratory animals approved by LAUTECH's animal care and use research ethical committee.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

I hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during writing or editing of manuscripts.

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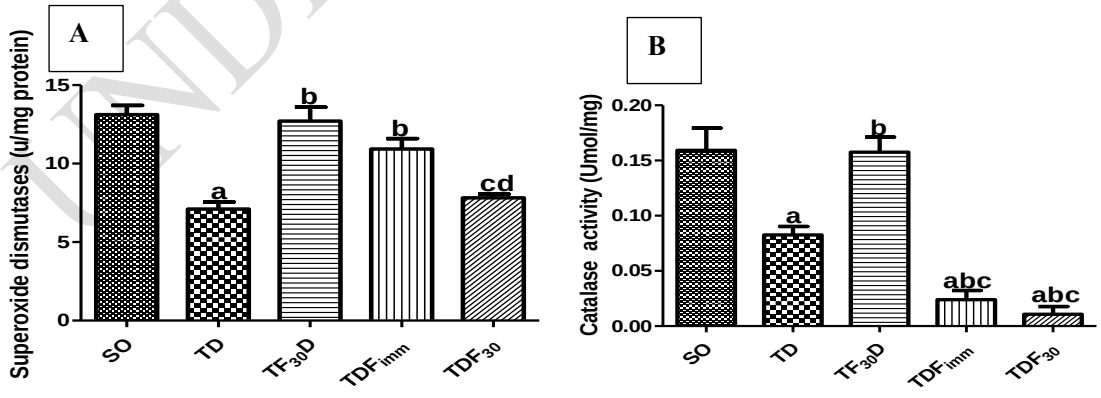
GRAPHS

Biochemical parameters



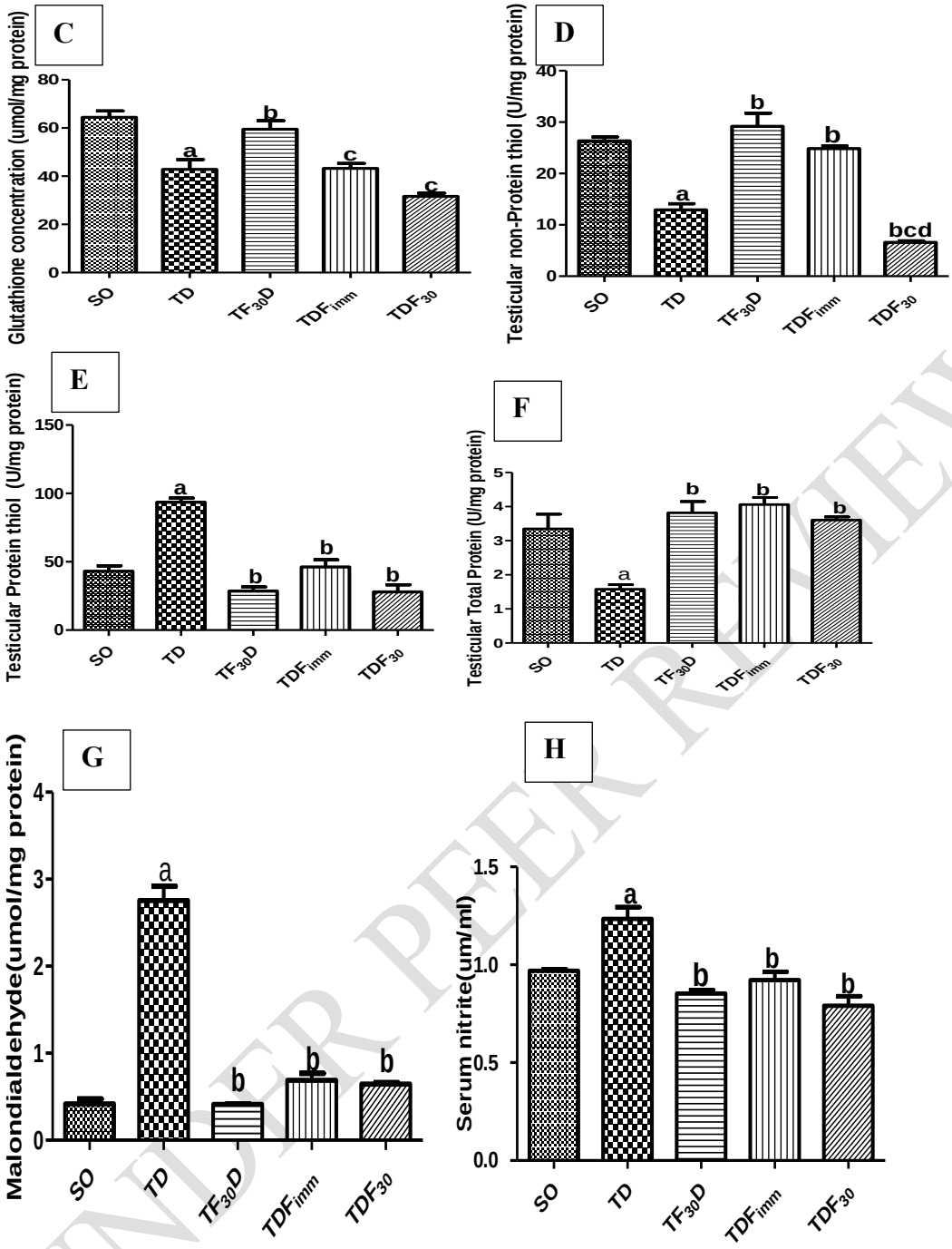


Figure 1A. The effect of timed administration of febuxostat on oxidative stress markers in male Wistar rats after 3 days of reperfusion

[a] represents significance at $p<0.01$ when compared to SO.
[b] represents significance at $p<0.05$ when compared to TD.
[c] represents significance at $p<0.001$ when compared to $TF_{30}D$.
[d] represents significance at $p<0.001$ when compared to $TFD_{imm.}$

Inflammatory markers

B

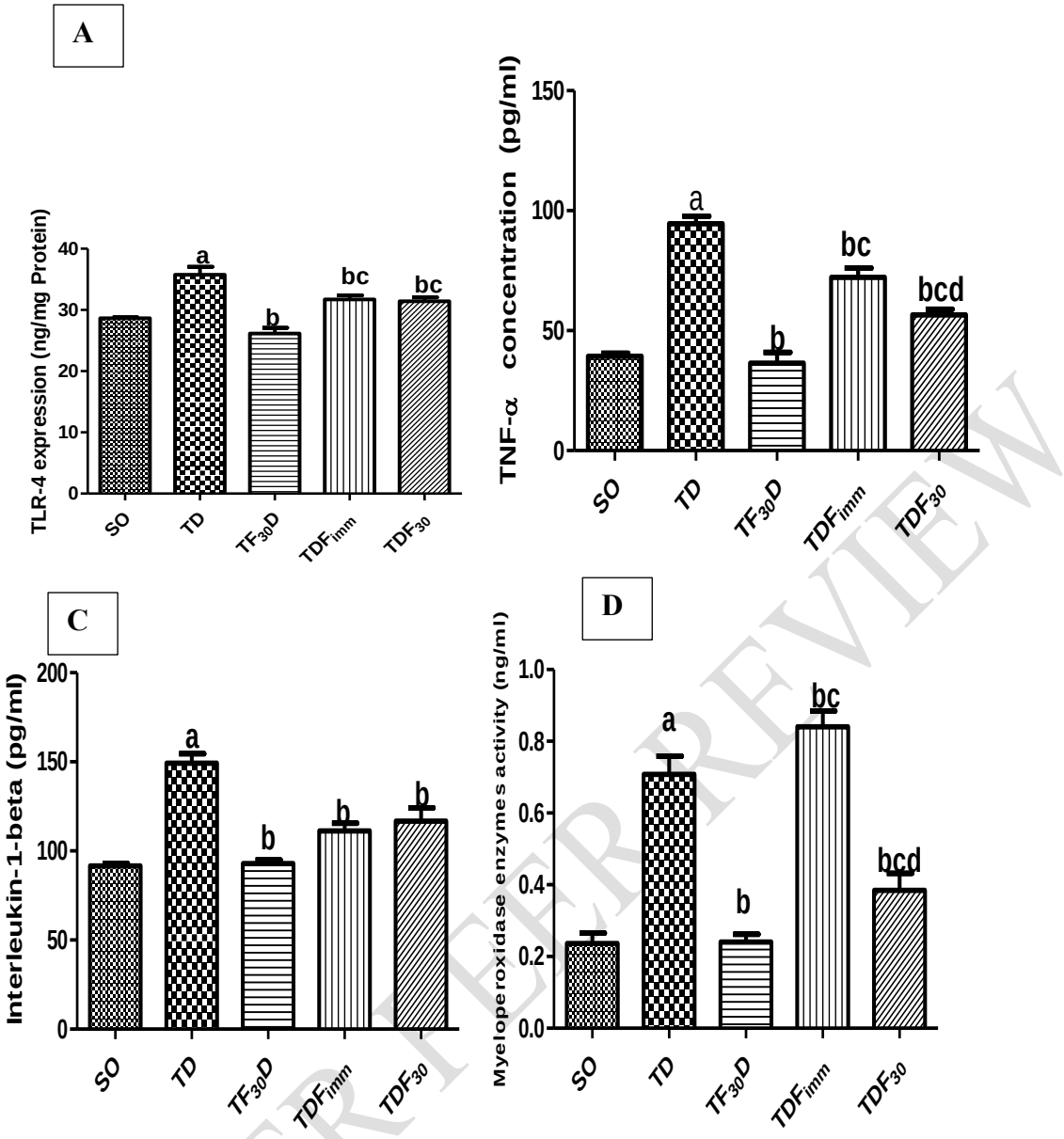


Figure 2A . The effect of timed administration of febuxostat on inflammatory markers in male Wistar rats after 3 days of reperfusion

[a] represents significance at $p<0.01$ when compared to SO.

[b] represents significance at $p<0.01;0.001$ when compared to TD.

[c] represents significance at $p<0.01;0.05$ when compared to $TF_{30}D$.

[d] represents significance at $p<0.05$ when compared to $TFD_{imm.}$

Reproductive hormones

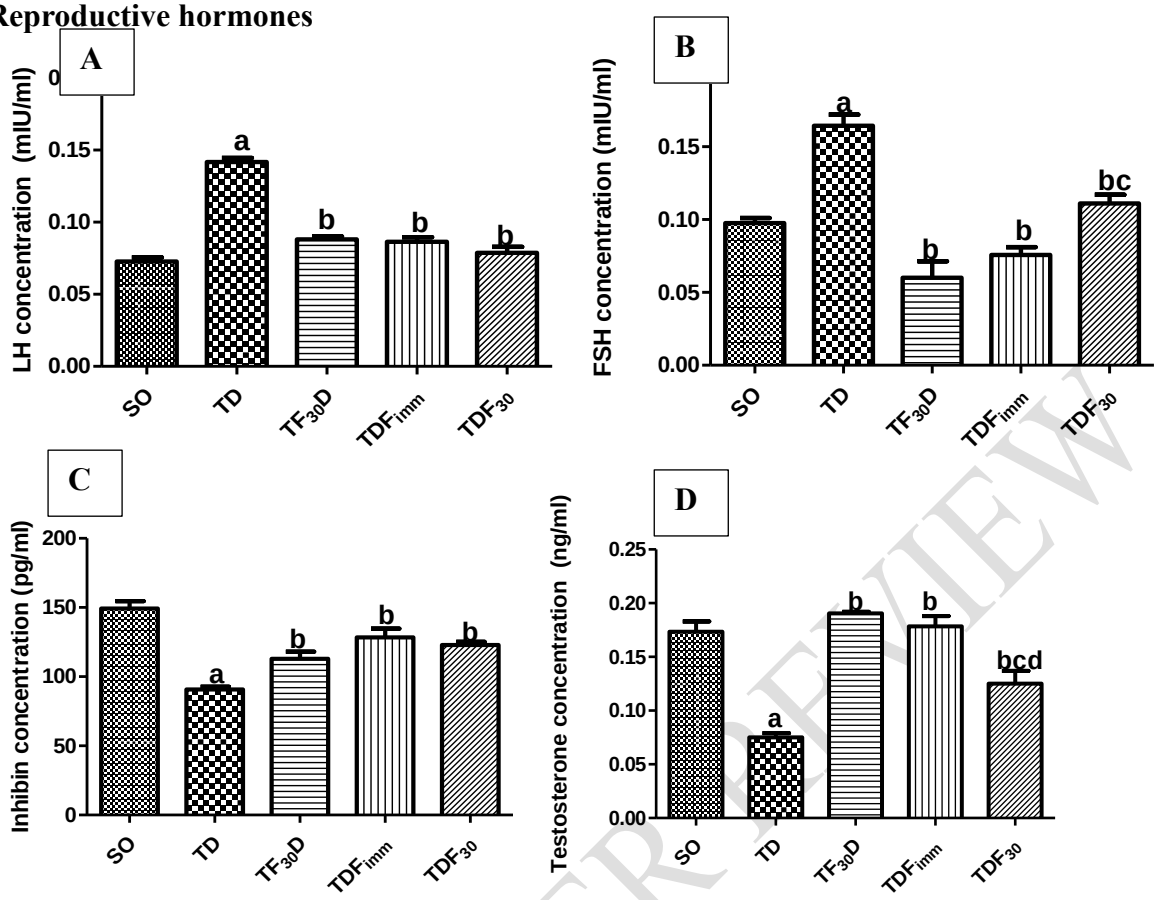


Figure 3A . The effect of timed administration of febuxostat on reproductive hormones in male Wistar rats after 3 days of reperfusion

[a] represents significance at $p<0.01$; 0.001 when compared to SO.

[b] represents significance at $p<0.01$;0.05; 0.001 when compared to TD.

[c] represents significance at $p<0.01$;0.05 when compared to $TF_{30}D$.

[d] represents significance at $p<0.05$ when compared to $TFD_{imm.}$