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Timed Administration of Febuxostat Improved Testicular Function Following Testicular Ischemia-reperfusion Injury Via Inhibition of MDA/NO Pathway, Down-regulation of Toll-like Receptor 4 Expression and Restoration of Reproductive Hormones

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Authors' contributions

This work was carried out in collaboration among all authors. 'All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: Testicular ischemia-reperfusion injury (TIRI) generates reactive oxygen species (ROS) through xanthine oxidase (XO) activity in the ischemic phase and leukocyte infiltration to the site of injury during reperfusion leading to oxidative stress, inflammation, and disruption of male 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 rats each): Group 1: Sham operated (SO) rats underwent surgery without TIRI induction, Group 2: Torsion + Detorsion (TD) rats underwent left unilateral TT for one hour and detorsed immediately to induce reperfusion which lasted for 3 days, Group 3: Torsion + 5 mg/kg Febuxostat + Detorsion ($TF_{30}D$), Group 4: Torsion + Detorsion + 5 mg/kg $Febuxostat_{imm}$ (TDF_{imm}) and Group 5: Torsion + Detorsion + 5 mg/kg $Febuxostat_{30}$ (TDF_{30}). $TF_{30}D$, TDF_{imm} and TDF_{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, Tumor-necrosis factor (TNF-α), interleukin-1-beta (IL-1β), 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) decreased antioxidant enzymes (SOD, CAT, GSH, non-protein thiols) and altering reproductive hormones (increased LH, FSH, and decreased testosterone and inhibin level) when compared to SO group ($p<0.01$; 0.001).

Febuxostat administered in the ischemic phase ($TF_{30}D$) significantly suppressed oxidative stress markers (MDA and serum nitrite), and improved antioxidant markers (SOD, CAT, TP, GSH, NPSH and PSH) when compared to TDF_{imm} and TDF_{30} groups ($p<0.001$). Additionally, all the febuxostat treated groups significantly reduced the level of TLR-4, TNF-α, and IL-1β, with MPO level only significantly reduced in $TF_{30}D$ and TDF_{30} groups ($p<0.001$;0.05) while the level of reproductive hormones (LH, FSH, inhibin, testosterone) were 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.

Keywords: Words; febuxostat; TIRI; time; inflammation; oxidative stress; antioxidant enzymes; reproductive hormones.

1. INTRODUCTION

Testicular ischemia-reperfusion injury (TIRI) is the interruption of blood flow to the testes followed by its restoration (Eltzschig & 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. Ghasemnejad-Berenji et al. (2017), it may result in testicular damage and eventually infertility in males if not promptly and properly managed. Activation of oxidative stress pathway and toll like receptor-4-induced inflammatory cascades has been documented to be involved in the etiology of TIRI-induced testicular damage (Molteni et al., 2016; Almarzouq & Al-Maghrebi, 2023).

The TIRI which is a two-phase process, involves the ischemic and reperfusion phases (Eltzschig & Eckle, 2011). The ischemic phase is characterized by production of xanthine oxidase (XO), a major reactive oxygen species (ROS)

generator (Shafik, 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-turn alters the integrity and permeability of the testicular cells (Tuncer et al*.,* 2007). In addition to this, there is massive ROS production during reperfusion due to 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., 2024; Marini et al., 2022). 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, 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 proteins (DAMP) are activated which triggers TLR-4. This 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 also increase intratesticular ROS and may inflict damage to the testes. Previous studies have further reported that ROS generated during TIRI affects reproductive hormones via attack on Leydig cell and Sertoli cells of the testes (Al-Maghrebi & Renno, 2016). Xanthine oxidase (XO) generated during the ischemic phase is the originator of ROS production (Shafik, 2012; Yu et al., 2023) and 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 may also contributes to ROS burst in the later phase (90-120 minutes) of reperfusion. Owing to this, 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. Study conducted by 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 Jr, 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, 2006) and gout but not in the management of TIRI. It has been reported to exhibit antioxidant (Rashad et al., 2023), anti-inflammatory (Amirshahrokhi, 2019), cytoprotective (Fahmi et al., 2016) and anti-apoptotic properties (Krishnamurthy et al., 2015). It reno-protective effect against renal ischemia-reperfusion injury in the kidney has previously been reported (Fahmi et al., 2016; El-Shoura et al., 2024). It also protects 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. 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*.

2.2 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.

2.3 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 + $Febuxostat_{30}$ (TDF_{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).

2.4 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, 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*.* (Afolabi et al., 2022).

2.5 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.

2.6 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.

2.7 Assessment of Testicular Weights

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

2.8 Biochemical Analysis

Testicular homogenates was used to assess superoxide dismutase (SOD) activity spectrophotometrically using the protocol of Paoletti et al*.,* (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, (Williams et al., 2014). Serum nitrite concentration was assessed by checking the nitrite level as described by Tatsch et al. (2011).

2.9 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.

2.10 Reproductive Hormones

Serum FSH, LH, inhibin and testosterone hormone concentration were measured from blood samples taken via retro-orbital puncture, allowed to clot 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).

2.11 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. 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 $P<0.05$) (Figs. 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) (Figs. 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) (Figs. 3A, B, C, and D).

3.1 Graphs

The graphs presented in this study showed the effect of timed administration of febuxostat on key testicular parameters following ischemia-reperfusion injury (TIRI).

3.1.1 Biochemical parameters

The biochemical markers presented below showed significant reductions in the biochemical parameters in TD group, indicating increased oxidative stress. Febuxostat-treated groups, especially $TF_{30}D$, demonstrated marked improvements in these antioxidant markers.

3.1.2 Inflammatory markers

TLR-4, TNF-α, and IL-1β, were significantly elevated in the TD group compared to the sham group, reflecting heightened inflammation. Febuxostat treatment, particularly in the $TF_{30}D$ group, significantly attenuated the levels of these markers, indicating its efficacy in reducing TIRI-induced inflammation.

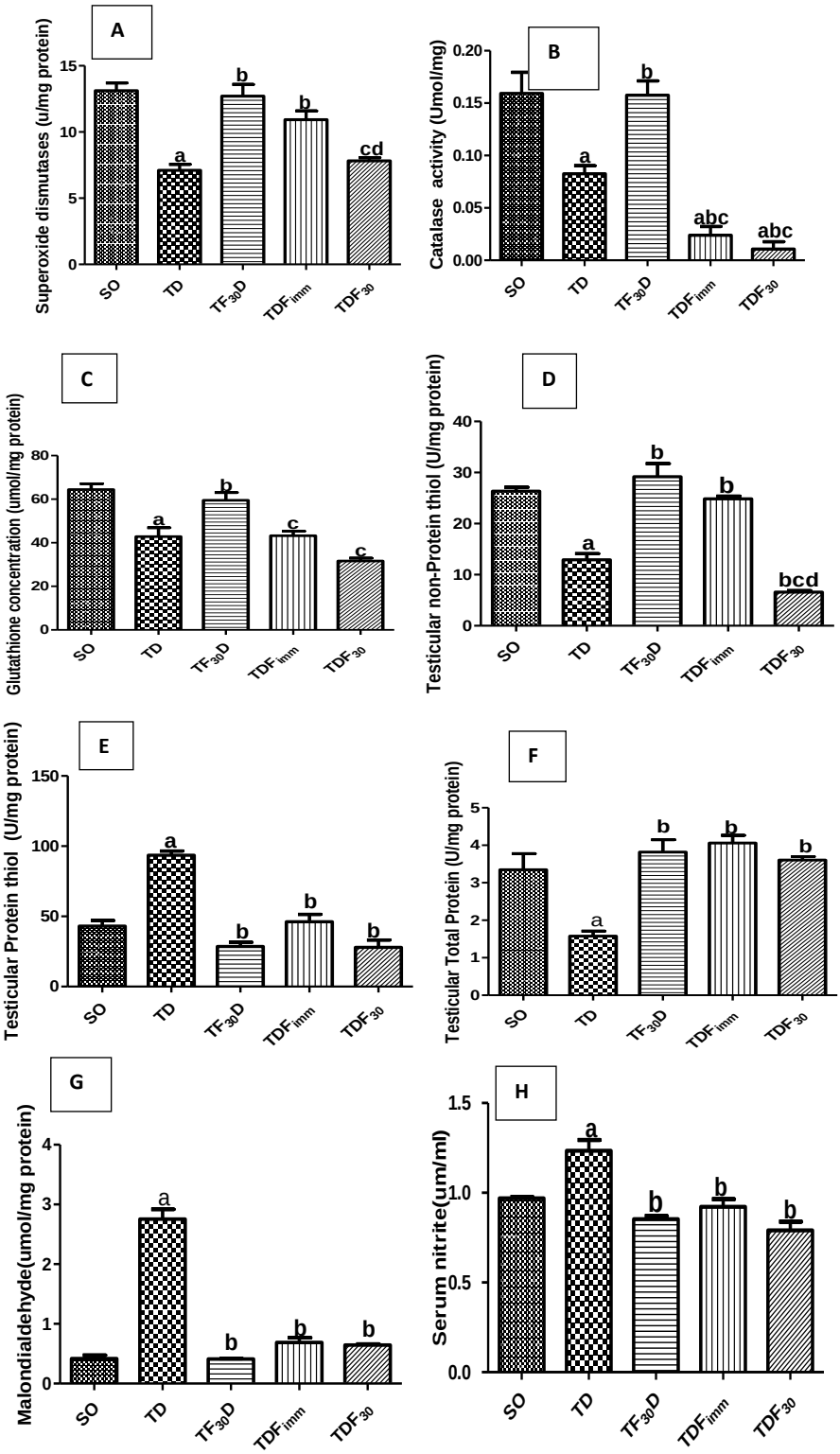

Fig. 1A. The effect of timed administration of febuxostat on oxidative stress markers in male Wistar rats after 3 days of reperfusion

[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}.

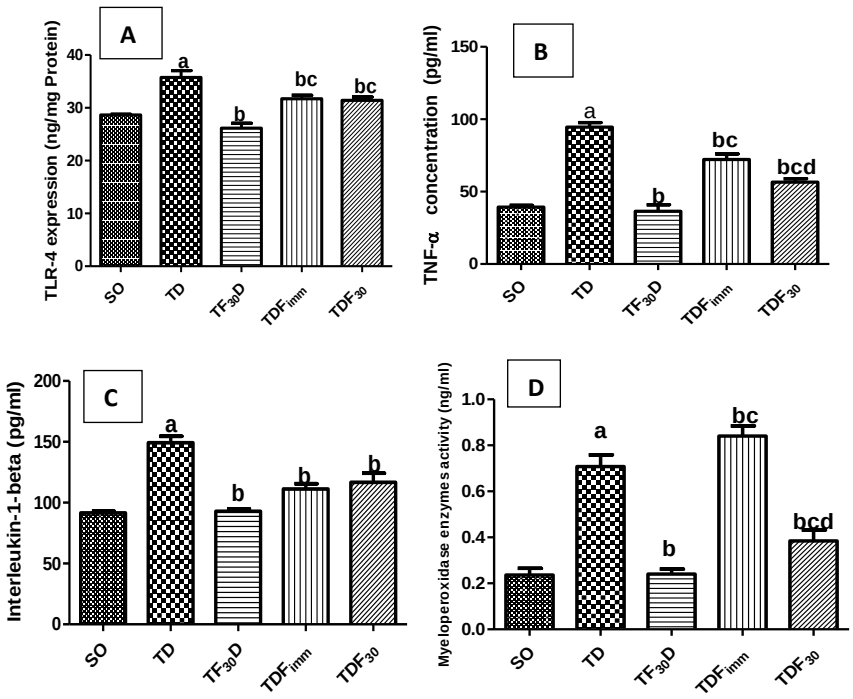

Fig. 2A. The effect of timed administration of febuxostat on inflammatory markers in male Wistar rats after 3 days of reperfusion

[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 $TFD_{imm.}$

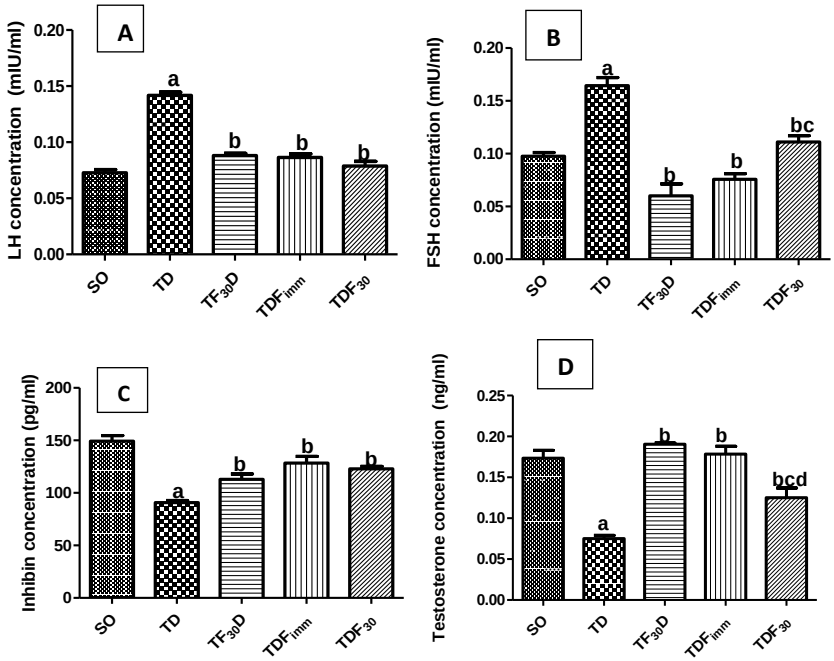

Fig. 3A. The effect of timed administration of febuxostat on reproductive hormones in male Wistar rats after 3 days of reperfusion

[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 $TFD_{imm.}$

3.1.3 Reproductive hormones

Reproductive hormone levels showed significant disruption in the TD group, with elevated LH and FSH, alongside reduced testosterone and inhibin levels. Febuxostat treatment, especially in the ischemic phase ($TF_{30}D$), restored hormonal balance, suggesting its potential to protect testicular function by mitigating hormonal dysregulation caused by TIRI.

4. 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 in massive ROS production that can up-regulate toll-like receptor activity 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). 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 & Akinloye, 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 (Tsaturyan et al., 2022). 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) (Circu & Aw, 2012 ; Kukurt et al., 2021).

In this study, the observed depletion in the antioxidant markers (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 when compared to SO group (Tsaturyan et al., 2022). Treatment with febuxostat at $TF_{30}D$ showed a superior effect in preventing oxidative stress than TDF_{imm} and TDF_{30} by blocking XO-ROS pathway to preserve the activities of SOD and catalase in the testicular tissue which have been reported in previous studies (Douzinas & Apeiranthitis, 2019).

Febuxostat, a potent XO inhibitor, could have exerted its protective effects by inhibiting the effect of ROS production in the ischemic phase 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 preserved endogenous thiol concentration following TD by preventing oxidation of cellular components that can be caused by XO-induced oxidative stress (Xu et al., 2008). The findings from this study also 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 lipid peroxidation with consequent decrease in MDA level (Kim et al., 2020). The surge in oxygen radicals after reperfusion 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 & 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 & 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 essential for sperm production and sexual behavior investigated in this study. Spermatogenesis, the process of sperm production, is tightly regulated by testosterone, inhibin and gonadotropins (Ramaswamy & Weinbauer, 2014; Anso, 2023). Inhibin, produced from the Sertoli cell of the testes helps to regulate FSH production which is crucial for spermatogenesis while testosterone, from the Leydig cell is involved in the entire process of sperm maturation. TIRI may disrupts spermatogenesis by disrupting the function of testicular cells (Kongmanas, 2008). Testosterone and inhibin synthesis in the testicular cells are controlled by the hypothalamus and pituitary gland through the action of FSH and LH and a negative feedback loop is constantly needed to maintain hormonal balance (McQuaid et al., 2014). Elevated levels of LH and FSH, alongside decreased inhibin and testosterone, suggest TIRI caused oxidative stress that may damage Leydig and Sertoli cells, thus impairing testicular function. Previous research has also demonstrated TIRI's adverse effects on Leydig and Sertoli cell function (Al-Maghrebi & Renno, 2016). Consistent with other studies, the observed rise in LH and decline in testosterone level in the TD group may be an indication of potential primary hypogonadism (Traish & Zitzmann, 2015).

Additionally, the observed increase in FSH, a key hormone in spermatogenesis, is an indication of disrupted sperm production (Chen et al., 2017). Kongmanas et al. (2008) report in their study that TIRI disrupts spermatogenesis. The disruption of Leydig and Sertoli cells impairs spermatogenesis via altered 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 hormone production. The overall hormonal imbalance caused by TIRI in this study suggests significant damage to the testicular cells, which may lead to impaired reproductive function. However, febuxostat raised the testosterone levels in this study, both in the ischemic phase (TF30D) and reperfusion phases (TDF_{imm} and TDF_{30}) which may be due to stimulation of epidermal growth factor in the Leydig cell, essential for cell proliferation and survival and previous study has confirmed this Damiani and Tiribelli (2023).

Increased testosterone levels in the febuxostat administered groups ($TF_{30}D$, TDF_{imm} and TDF_{30}) is the reason for the observed restoration of luteinizing hormone (LH) to normal level after TIRI. The restoration of LH to normal level is due to activation of negative feedback mechanism by increased testosterone level to restore LH level (Pitteloud et al., 2008). Testosterone and follicle-stimulating hormone (FSH) work in tandem to stimulate spermatogenesis in the seminiferous tubules of the testes. In this study, restoration of FSH levels to normal in the $TF_{30}D$, TDF_{imm}, and TDF_{30} groups suggests restoration of hormonal balance and improved spermatogenesis (Santi et al., 2020). Sertoli cells play a major role in spermatogenesis by secreting inhibin, which regulates FSH production. Inhibin enhances mitotic activity of spermatogonia and provides structural and nutritional support to germ cells (Griswold, 2018). The increased inhibin levels in $TF_{30}D$, TDFimm, and TDF_{30} indicate improved Sertoli cell activity and enhanced spermatogenesis. Additionally, inhibin regulate FSH level via its action on the pituitary gland (Makanji et al., 2011). The administration of febuxostat during the ischemic ($TF_{30}D$) and reperfusion (TDFimm, TDF_{30}) phases appears to reduce the effect of reactive oxygen species (ROS) on testicular cells and thus, regulate hormone balance and enhance testicular function (Al-Maghrebi et al., 2020; Marini et al., 2022).

5. CONCLUSION AND RECOMMENDATION

Treatment with febuxostat at $TF_{30}D$ has shown promising effects in improving antioxidants, reducing oxidative stress and inflammation. Also,

administration of febuxostat at $TF_{30}D$ restore hormonal function and prevent damage to testicular cells to enhance spermatogenesis. Febuxostat administration during ischemic phase ($TF_{30}D$) should be explored as a therapeutic strategy to improve testicular function against xanthine oxidase-driven ROS production in TIRI. Further studies are recommended to optimize the dosage of febuxostat to maximize its protective effects against TIRI.

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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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