Biomedicine & Pharmacotherapy 133 (2021) 111086

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Proxeed plus salvage rat testis from ischemia- reperfused injury by enhancing antioxidant's activities and inhibition of iNOS expression

Janet Olayemi Sangodele [a,*], Zephaniah Inuwa [a], Bashir Lawal [b], Grace Adebayo-Gege [c], Bamidele Joseph Okoli [d,e], Fanyana Mtunzi [d]

[a] Department of Biochemistry, Bingham University, Abuja-Keffi Expressway Road, P.M.B 005, Karu, Nigeria
[b] Graduate Institute for Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taiwan
[c] Department of Human Physiology, Faculty of Basic Medical Sciences, Baze University, Abuja, Nigeria
[d] Institute of Chemical and Biotechnology, Vaal University of Technology, Science Park, Private Bag x021, South Africa
[e] Department of Chemical Sciences, Faculty of Science and Technology, Bingham University Karu, Nigeria

ARTICLE INFO

Keywords:
Proxeed plus
Epididymis
Testicular ischemia
Apoptosis
Sex hormones
Testicular damage

ABSTRACT

Testicular torsion is an acute urological emergency condition that occurs due to obstruction of blood flow to the testicles which may result in ischemia and loss of testicular functions. This study examined the protective effects of Proxeed Plus (PP), a dietary supplement on testicular ischemia/reperfusion (I/R) injured rats using oxidative stress markers, hormonal levels, apoptotic parameters, histological and immunohistochemistry analysis at 4 h and after 7 days of reperfusion. The protective treatment of the I/R injured rats with PP at 1000 and 5000 mg/kg body weight (bw) resulted in significant increases in the serum and tissue antioxidative defense capacities (superoxide dismutase, reduced glutathione, catalase, glutathione-s-transferase, and glutathione peroxidase), sex hormones (luteinizing hormone, follicle-stimulating hormone, and testosterone), also reduce pro-oxidative markers (malondialdehyde and hydrogen peroxide), serum iNOS and apoptotic parameters (Caspase -3 and Caspase -9) in comparison to the results detected in the I/R untreated rats. It was also observed that PP ameliorated histological changes of I/R injured rats; increased spermatogenetic activity, seminiferous tubular diameter, Leydig cell mass, and reduced expressions of testicular inducible nitric oxide synthase (iNOS). Therefore, the therapeutic use of Proxeed Plus could be considered a promising approach in averting testicular damage against I/R injury.

1. Introduction

Ischemia-reperfusion injuries are associated with fatal clinical manifestations, such as cerebral ischemic stroke [1,2], hibernating myocardium, acute cardiac failure, acute myocardial infarction [3], systemic inflammatory response syndrome, gastrointestinal dysfunction, and multi-organ dysfunction syndrome. Over productions of free radicals is one of the major pathophysiological responses of ischemia-reperfusion injuries, during ischemia, the flow of blood is restricted for a certain period, while during reperfusion, the blood flow is restored and oxygen enters the tissue [4] causing over production of reactive oxygen species (ROS) and decrease levels of antioxidants in ischemic cells [5]. These ROS induces oxidative stress that enhances organs dysfunction, DNA damage, and local inflammatory responses. Inflammatory cascades and oxidative stress may subsequently induce a cytokine storm, damage to cellular structures and cell death [6].

Testicular torsion is otherwise known as the spermatic cord rotation around its axis; it obstructs blood flow to the testicles, leading to a shortage of oxygen supply and loss of testicular function [7]. It is an acute pediatric urological emergency with an incident rate of 1 in 4000 males especially childhood and adolescence below the age of 25 [8]. Early detections and treatment are essential because, testicle torsed for longer than 6 h are considered out of survival period. About 90–100 % of testicles can be salvaged if treatment is administered within the survival period, 20–50 % of testicles can be salvaged if treatment is administered within 6–12 h [9], however, treatment within 12–24 h, of torsion could only yield maximum of 10 % survival rate [10]. Surgical detorsion treatment has yielded 42–88 % survival rate, but whether these testes

* Corresponding author.
E-mail addresses: janetolayemi@binghamuni.edu.ng (J.O. Sangodele), kaigamazeph1@gmail.com (Z. Inuwa), bashirlawal12@gmail.com (B. Lawal), funbimbola@gmail.com (G. Adebayo-Gege), okolibj@binghamuni.edu.ng (B.J. Okoli), fanyana@vut.ac.za (F. Mtunzi).

https://doi.org/10.1016/j.biopha.2020.111086
Received 14 July 2020; Received in revised form 19 November 2020; Accepted 28 November 2020
Available online 11 December 2020

were rescued from the ischemic induced spermatogenic abnormalities remain poorly understood [9,11].

Epidemiological and experimental studies have reported the association between male infertility and oxidative stress [12]. Over productions of free radicals as one of the major pathophysiological responses of testicular ischemia-reperfusion (I/R) injury during testicular torsion lead to oxidative stress which in turn compromises the integrity of seminiferous epithelium [13] and finally result to sterility in adult male. The spermatozoa are highly susceptible to these oxidative attacks due to the high levels of polyunsaturated fats (PUFAs) and limited cytoplasm antioxidant enzymes [14]. These enhance lipid peroxidation (LPO), DNA fragmentation, and consequently impaired spermatogenesis and sperm motility [14,15]. To date, researchers have attempted numerous anti-inflammatories, antioxidants, and radical scavengers to attenuate testicular I/R injury and facilitate spermatogenesis [16–18]. However, pleasing preclinical and clinical research regarding the treatment of testicular torsion is missing in the literature therefore, studies with regards to this challenge is of increasingly welcome.

Studies using several experimental investigations have demonstrated the potential of antioxidant-rich supplements in the management of ischemia/reperfusion injury in animals [19,20]. Proxeed Plus is a dietary supplement, specifically formulated to optimize sperm health. It consists of L- carnitine, acetyl- L- carnitine, zinc, fumarate, CoQ10, folic acid, fructose, vitamin C, and vitamin B12 as the active ingredient [21]. These ingredients are known to play a vital function in spermatozoa energy metabolism, hormonal metabolism, antioxidant defense systems and immune function [22–24]. It has been documented for nephroprotective against toxicant induced nephrotoxicity [25]. The growing speculation about the antioxidant capability of Proxeed Plus worldwide, supported by the fact that no study has been carried out with the intent of determining the protective role of Proxeed Plus against ischemia-reperfusion injury has given rise to a need to evaluate these potentials. Thus, the present study investigated the protective effect of Proxeed Plus on testis experimentally-induced I/R injury in male Wistar rats.

2. Materials and methods

2.1. Test supplement (proxeed plus)

The test supplement (Proxeed Plus) was obtained from Sigma-Tau Health Science, Utrecht, the Netherlands. It consists of 1000 mg L-carnitine, 500 mg acetyl- L- carnitine, 10 mg zinc, 725 mg fumarate, 20 mg CoQ10, 200 μg folic acid, 1000 mg fructose, 90 mg vitamin C, and 1.5 μg vitamin B12.

2.2. Experimental animals

Fifty (50) male albino rats weighing (155.87 ± 7.65 g) were procured from the Animal House of Bingham University, Karu, Nigeria. The animals were maintained under standard laboratory conditions with access to food and water ad libitum. The rats were housed in standard cages at 22 °C (50 % humidity) with a 12 -h light-dark cycle. The animals fasted for 12 h before the commencement of any study.

2.3. Experimental method

The animals were randomized into 5 (n = 6). Group 1 was assessed as the control group. Group 2 was assessed as the Sham group. Group 3 was assessed ischemia-reperfusion (I/R) group. In Group 3, testicular torsion was first performed, followed by testicular detorsion and induction of reperfusion. In Group 4 and Group 5, following testicular torsion and detorsion, Proxeed Plus was administered at doses of 1000 mg/kg bw and 5000 mg/kg bw respectively before inducing reperfusion. The animals were sacrificed after 4 h while treatments continue for 7 days for the rest of the animals in the groups. The surgical procedure was carried out based on previous experimental studies [26,27]. In brief, the rats were anesthetized using intraperitoneal injections of 50 mg/kg bw of ketamine hydrochloride, and 10 mg/kg bw xylazine. Proxeed Plus was dissolved in water and administered orally. The doses and means of administrations were carefully selected to make a reasonable comparison with human administration. Through a longitudinal scrotal incision, the left testis of the animals in each group was exposed and dissected. Afterward, the torsion of the left testis was induced by a 720° counter-clockwise rotation. One hour later, the testis was counter-rotated to the natural position and was inserted into the scrotum. Then, the skin incision was sutured (4–0 non-absorbable), Proxeed plus was administered at doses of 1000 mg/kg bw and 5000 mg/kg bw for 4 h and 7 days, and animals were kept until harvesting time. In the sham-operated animals, only surgical stress was applied by immediately retracting and replacing the spermatic cord. All animal experiments were carried out per the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European communities' council directive of 24 November 1986 (86/609/EEC) and the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). All experimental protocols were approved by Bingham University, Committee on Ethics for Medical and Scientific Research (BHU/REC/19/A005), and principles governing the use of laboratory animals as laid out by the Bingham University, Committee on Ethics for Medical and Scientific Research was duly observed.

2.4. Collection of blood, preparation of serum and hormonal estimation

The blood samples were collected in plain sample bottles and centrifuged at 3000 r.p.m. for 15 min [28–30], after which the serum was transferred into a plain sample bottle. Serum levels of Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), and testosterone were assayed by enzyme immunoassay (SIA) using the assay kits (HySkill Diagnostics, Bahlingen, Germany) as described by the manufacturer [31]. Absorbance was measured at 450 nm. The within-assay coefficient of variation was 6.1 % for FSH, 5.4 % for LH, and 6.2 % for testosterone. The analytic sensitivities of the assays were 1.0 mIU/mL for LH and FSH and 0.1 ng/mL for testosterone as provided by the manufacturer.

2.5. Analysis of biochemical parameters

The epididymis was homogenized in phosphate buffer (pH 7.4) and centrifuged at 10,000 net grams for 10 min at 4 °C and the supernatants were used for estimation of biochemical parameters using standard protocols. The collection of serum for analysis of the biochemical parameters was as described in Section 2.4. Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substance (TBARS) as described by Placer et al. [32]. The levels of TBARS were monitored at 532 nm by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane.

Glutathione peroxidase (GPX) catalyzed the oxidation of glutathione by cumene hydroperoxide. In presence of NADPH and glutathione reductase, the oxidized glutathione was immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$ [33]. Spectrophotometric methods was used to analyze the GPx and GST activities. To evaluate GPX activity in hemolysates 10 μL of samples were mixed with 500 μL mixed reagent and 20 μL cumene hydroperoxide. The absorbance was measured at 340 nm

Superoxide dismutase (SOD) activity was assessed based on the superoxide radicals production by xanthine and xanthine oxidase, which reacts with 2-(4-iodophenyl)-3-(4- nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye [34]. Briefly, 200 μL of diluted sample was mixed with 300 μL of the substrate followed by addition of 75 μL xanthine oxidase. The change in absorbance was measured at 505

nm

The levels of reduced glutathione (GSH) were assessed following the method of Ellman [35]. The procedures involve the reactions of thiols with Ellman's reagent (5,5′-dithiobis-(2- nitrobenzoic acid) or DTNB), via cleave of disulfide bond to yield 2-nitro-5-thiobenzoate (TNB^-), which ionizes to the TNB^{2-} dianion in water at alkaline and neutral pH.

Spectrophotometric methods as described by Koroliuk et al. [36] were used for the estimation of Catalase (CAT) activity. The sample (10 μL) was incubated for 10 min with 100 μmol/mL H_2O_2 in 0.05 mmol/L Tris−HCl buffer (pH = 7). The yellow complex of ammonium molybdate ($(NH4)_2MoO_4$) and H_2O_2 was monitored at 410 nm after terminating the reaction with 50 μL of 4 % $(NH4)_2MoO_4$. The protein concentrations by the method of Gornal et al. [37]. Serum iNOS levels was measured using ELISA detection kit (Shanghai LZ, China) according to the manufacturer's instruction.

2.6. Measurement of apoptosis

For the evaluation of apoptosis, Caspase-3 and Caspase-9 activities were measured using ELISA detection kit (Shanghai LZ, China) according to the Biotin double-antibody sandwich technology as described previously [38].

2.7. Histological and immunohistochemistry analysis

Bouin solution was used to fix the testes for 24 h. Staining was done with H&E to verify histological details. Mean testicular biopsy score (MTBS) described by Johansen [39], was used to rate testicular injury and spermatogenesis. For the immunohistochemistry studies, paraffin sections were deparaffinized in xylene and processed for iNOS antibody immunohistochemistry as described by Al-Drees et al. [40].

2.8. Mean seminiferous tubular diameter and Leydig cell mass

The mean seminiferous tubular diameter (MSTD) was determined by measuring 20 separate round seminiferous tubules with a light microscope-adaptable micrometer for each testis. The number of germ cell layers in each tubule for each testis was determined by counting the number of germ cell layers from the basement membrane to the lumen at 90°, 180°, 270°, and 360° and averaging these numbers. Procedures described by Roosen-Runge [41] was adopted for scoring the number of Leydig cell in each tubule

2.9. Data analysis

Data were expressed as the Mean ± SD of six determinations. All data generated follow normal distribution. The analysis was performed using the SPSS statistical package for Windows (version 21.0; SPSS Inc, Chicago). Results were subjected to ANOVA followed by DMRT. Statistically significant was consider at $p < 0.05$

3. Results

3.1. Effects of Proxeed Plus on serum and tissue antioxidants parameters

The serum levels of GSH, GST, CAT, SOD, and GPx were significantly lower in IR rats in comparison to the Control, Sham group and PP treated rats at 4 h and 7 days of treatments. Treatment with PP produced dose-dependent increases in the antioxidant parameter (Table 1 and 2). Similarly, GSH levels, GST, CAT, SOD and GPx activities in rats administered 5000 mg/kg bw PP were significantly ($p < 0.05$) higher than that of the Control group. However, the Sham group exhibited lower SOD activity and GSH level when compared with the control (Tables 3,4).

3.2. Effects of proxeed plus on protein, MDA levels and H_2O_2 generation on I/R injured rats

The serum MDA levels and hydrogen peroxide generation were significantly higher while proteins was lower in I/R rats in comparison

Table 1
Effects of 4 h Treatments with Proxeed Plus on serum antioxidants enzymes on ischemia-reperfusion injury in male rats.

	4 h Treatments				
	Catalase (μmolH_20_2/min/mgproten)	SOD (units/mgprotein)	GSH (μmol/mgprotein)	GPX (units/mgprotein)	GST (μmole CDNB–GSH complex formed/min/mg protein)
Control	123.52 ± 11.58 ^{b}	2.50 ± 0.23 ^{c}	69.52 ± 7.03 ^{c}	11.71 ± 3.60 ^{ab}	0.17 ± 0.07 ^{bc}
Sham	118.57 ± 12.77 ^{b}	1.25 ± 0.30^{b}	53.80 ± 9.58 ^{b}	11.84 ± 0.39 ^{ab}	0.12 ± 0.06 ^{b}
I/R	108.28 ± 15.34^{a}	0.80 ± 0.20 ^{a}	6.73 ± 0.84 ^{a}	10.51 ± 0.98 ^{a}	0.07 ± 0.01 ^{a}
I/R + 1000 mg/kg PP	126.92 ± 7.32 ^{b}	2.66 ± 0.22 ^{c}	70.68 ± 7.86 ^{c}	12.12 ± 0.39 ^{b}	0.20 ± 0.04 ^{c}
I/R + 5000 mg/kg PP	132.11 ± 7.32 ^{c}	3.53 ± 0.22 ^{d}	84.42 ± 4.24 ^{d}	13.51 ± 1.89 ^{b}	0.38 ± 0.09 ^{d}

Values are expressed as Mean ± SD of 6 determinations.
I/R: Ischemia/Reperfusion; PP: Proxeed Plus; CAT: Catalase (μmolH_20_2/min/mgproten), GSH: Reduced glutathione (μmol/mgprotein), GST: glutathione S-transferase (μmole CDNB–GSH complex formed/min/mgprotein), SOD: superoxide dismutase (units/mgprotein), and GPx: glutathione peroxidase (units/mgprotein).

Table 2
Effects of 7 days h Treatments with Proxeed Plus on serum antioxidants enzymes on ischemia-reperfusion injury in male rats.

	7 Days Treatments				
	Catalase (μmolH_20_2/min/mgproten)	SOD (units/mgp rotein)	GSH (μmol/mgpr otein)	GPX (units/mgprotei n)	GST (μmole CDNB–GSH complex formed/min/mgprotein)
Control	123.52 ± 11.58^{ab}	2.50 ± 0.23^{b}	69.52 ± 7.03^{c}	11.71 ± 3.60^{a}	0.17 ± 0.07^{a}
Sham	118.57 ± 12.77^{a}	1.25 ± 0.30^{a}	53.80 ± 9.58^{b}	11.84 ± 0.39^{a}	0.12 ± 0.06^{a}
I/R	111.54 ± 21.69^{a}	1.15 ± 0.85^{a}	38.23 ± 6.03^{a}	11.03 ± 0.41^{a}	0.09 ± 0.03^{a}
I/R + 1000 mg/kg PP	138.77 ± 4.29^{b}	3.55 ± 0.35^{c}	72.10 ± 7.58^{c}	14.02 ± 0.54^{b}	0.81 ± 0.94^{c}
I/R + 5000 mg/kg PP	160.53 ± 5.52 ^{c}	5.15 ± 0.25^{d}	84.89 ± 6.06^{d}	16.41 ± 1.44^{b}	1.48 ± 0.97^{d}

Values are expressed as Mean ± SD of 6 determinations.
I/R: Ischemia/Reperfusion; PP: Proxeed Plus; CAT: Catalase (μmolH_20_2/min/mgproten), GSH: Reduced glutathione (μmol/mgprotein), GST: glutathione S-transferase (μmole CDNB–GSH complex formed/min/mgprotein), SOD: superoxide dismutase (units/mgprotein), and GPx: glutathione peroxidase (units/mgprotein).

Table 3
Effects of 4 h Treatments with Proxeed Plus on epididymis GSH levels, GST, CAT, SOD and GPx activities on ischemia-reperfusion injury in male rats.

	4 h Treatments				
	Catalase (μmolH_2O_2/min/mgproten)	SOD (units/mgprotein)	GSH (μmol/mgprotein)	GPX (units/mgprotein)	GST (μmole CDNB–GSH complex formed/min/mgprotein)
Control	413.42 ± 14.3^{b}	0.90 ± 0.20 ^{a}	44.89 ± 3.17^{b}	45.07 ± 0.17^{b}	0.08 ± 0.03 ^{a}
Sham	412.21 ± 23.0^{b}	0.80 ± 0.27 ^{a}	43.77 ± 3.08^{b}	44.65 ± 4.69^{b}	0.07 ± 0.02 ^{a}
I/R	308.45 ± 10.6^{a}	0.10 ± 0.27 ^{a}	35.61 ± 9.95^{a}	30.21 ± 9.82^{a}	0.07 ± 0.02 ^{a}
I/R + 1000 mg/kg PP	480.97 ± 53.6^{b}	1.45 ± 0.05 ^{b}	47.14 ± 3.18^{b}	49.93 ± 0.37^{c}	0.10 ± 0.03 ^{ab}
I/R + 5000 mg/kg PP	545.36 ± 29.7^{c}	3.55 ± 0.95 ^{c}	60.20 ± 5.77^{c}	50.17 ± 4.12^{c}	0.12 ± 0.03 ^{b}

Values are expressed as Mean ± SD of 6 determinations.
I/R: Ischemia/Reperfusion; PP: Proxeed Plus; CAT: Catalase (μmolH_2O_2/min/mgproten), GSH: Reduced glutathione (μmol/mgprotein), GST: glutathione S-transferase (μmole CDNB–GSH complex formed/min/mgprotein), SOD: superoxide dismutase (units/mgprotein), and GPx: glutathione peroxidase units/mgprotein).

Table 4
Effects of 7 days of Treatments with Proxeed Plus on epididymis GSH levels, GST, CAT, SOD and GPx activities on ischemia-reperfusion injury in male rats.

	7 Days Treatments				
	Catalase (μmolH_2O_2/min/mgproten)	SOD (units/mgprotein)	GSH (μmol/mgpr otein)	GPX (units/mgprotei n)	GST (μmole CDNB–GSH complex formed/min/mgprotein)
Control	413.42 ± 14.31^{b}	0.90 ± 0.2^{b}	44.89 ± 3.17^{a}	45.07 ± 0.17^{b}	0.08 ± 0.03^{a}
Sham	412.21 ± 23.06^{b}	0.80 ± 0.2^{b}	43.77 ± 3.08^{a}	44.65 ± 4.69^{b}	0.07 ± 0.02^{a}
I/R	373.42 ± 18.90^{a}	0.35 ± 1.4^{a}	41.53 ± 3.46^{a}	38.94 ± 10.40^{a}	0.07 ± 0.03^{a}
I/R + 1000 mg/kg PP	592.43 ± 31.49^{c}	4.60 ± 4.2^{c}	68.77 ± 9.59^{b}	55.97 ± 2.56^{c}	0.12 ± 0.07^{b}
I/R + 5000 mg/kg PP	614.70 ± 31.50c	5.20 ± 0.8^{d}	83.87 ± 4.84^{c}	57.79 ± 2.56 ^{c}	0.17 ± 0.11^{c}

Values are expressed as Mean ± SD of 6 determinations. I/R: Ischemia/Reperfusion; PP: Proxeed Plus; CAT: Catalase (μmolH_2O_2/min/mgproten), GSH: Reduced glutathione (μmol/mgprotein), GST: glutathione S-transferase (μmole CDNB–GSH complex formed/min/mgprotein), SOD: superoxide dismutase (units/mgprotein), and GPx: glutathione peroxidase (μmol/mgprotein).

to the control. Rats in the Sham group and those treated with PP at 4 h and 7 days had lower MDA levels and H_2O_2 generation than the I/R. At 7 days of treatment, serum protein concentration was higher in rats dosed 5000 mg/kg bw PP when compared with the control and other experimental groups (Table 5).

The MDA levels and H_2O_2 generations in the epididymis were significantly higher in I/R rats in comparison to the control. Treatment with PP at 4 h and 7 days decrease ($p < 0.05$) the MDA levels and H_2O_2 generations when compared with the I/R group. The MDA levels and H_2O_2 generations were lower in rats treated with the 5000 mg/kg bw of PP than the Sham and control group. At 4 h and 7 days treatment, the protein concentration in epididymis was higher in rats treated with 5000 mg/kg bw PP than the I/R, sham and control group (Table 6).

3.3. Effects of Proxeed Plus on hormonal levels in I/R injured rats

The serum levels of LH and testosterone in I/R rats were significantly ($p < 0.05$) lowered in comparison to the control, sham group and PP treated rats. Moreover, in the group treated with 5000 mg/kg PP, the LH and testosterone levels were higher than that of the control and sham group ($p < 0.05$). Similarly, serum level of FSH in control, sham, and PP treated rats after 7 days of treatment was significantly ($p < 0.05$) higher than the I/R rats (Table 7)

3.4. Effects of Proxeed Plus on activities of iNOS and apoptotic markers of I/R injured rats

Caspase-3, Caspase-9 and iNOS were higher in the I/R group than those in the Control, Sham and PP treated groups. At 4 h of treatments,

Table 5
Effects of Proxeed Plus on serum Protein, MDA levels and hydrogen peroxide generation on ischemia-reperfusion injury in male rats.

	4 h Treatments			7days Treatments		
	Protein (g/dl)	MDA (units/g tissue)	H_2O_2 (nmol/mg protein)	Protein (g/dl)\	MDA (units/g tissue)	H_2O_2 (nmol/mgprotein)
Control	3.57±0.08^{b}	1.97±0.12^{ab}	151.50±8.83^{a}	3.57±0.08^{a}	1.97±0.12 ^{a}	151.50±8.83^{b}
Sham	3.44±0.07^{b}	2.21±0.42^{b}	178.91±4.19^{b}	3.44±0.07^{a}	2.21±0.42^{b}	178.91±4.19^{c}
IR	2.88±0.62^{a}	5.57±0.40^{c}	190.83±0.77^{c}	3.20±0.76^{a}	3.44±0.07^{c}	185.16±7.20^{d}
I/R+1000mg/kg PP	3.66±0.17^{b}	1.44±0.35^{a}	181.09±5.11^{b}	3.62±0.12^{a}	1.34±0.37^{a}	168.33±17.76^{b}
I/R+5000mg/kg PP	3.71±0.22^{b}	1.21±0.54^{a}	178.62±6.37^{b}	4.14±0.34^{b}	1.03±0.81^{a}	144.50±9.41^{a}

Values are expressed as Mean ± SD of 6 determinations. I/R: Ischemia/Reperfusion; PP: Proxeed Plus.

Table 6
Effects of Proxeed Plus on epididymis Protein, MDA levels and hydrogen peroxide generation on ischemia-reperfusion injury in male rats.

		4 h Treatments			7 days Treatments	
	Protein (g/dl)	MDA (units/g tissue)	H_2O_2 (nmol/mg protein)	Protein (g/dl)	MDA (units/g tissue)	H_2O_2 (nmol/mg protein)
Control	1.01 ± 0.07^{b}	1.97 ± 0.12 ^{b}	35.16 ± 3.21^{a}	1.01 ± 0.07^{a}	1.97 ± 0.12 ^{a}	35.16 ± 3.21^{b}
Sham	0.99 ± 0.04^{b}	2.21 ± 0.42^{b}	39.87 ± 5.62^{a}	0.99 ± 0.04^{a}	2.21 ± 0.04^{b}	39.87 ± 5.62^{b}
IR	0.85 ± 0.08^{a}	5.57 ± 0.40 ^{c}	93.50 ± 7.00^{b}	0.92 ± 0.32^{a}	3.44 ± 0.07 ^{c}	51.58 ± 3.21^{c}
I/R + 1000 mg/kg PP	1.06 ± 0.14^{b}	1.44 ± 0.35 ^{a}	42.00 ± 7.75^{a}	2.97 ± 0.98^{b}	1.34 ± 0.37^{a}	36.50 ± 1.00 ^{b}
I/R + 5000 mg/kg PP	1.50 ± 0.02^{c}	1.21 ± 0.54 ^{a}	40.87 ± 3.62^{a}	4.01 ± 0.48^{c}	1.03 ± 0.81^{a}	22.75 ± 3.00 ^{a}

Values are expressed as Mean ± SD of 6 determinations. I/R: Ischemia/Reperfusion; PP: Proxeed Plus.

Table 7
Effects of Proxeed Plus on LH, FSH, Testosterone in Ischemia-Reperfusion male rats.

	4 h Treatments			7 days Treatments		
	LH (mIU/mL)	FSH (mIU/mL)	TESTOSTERONE (ng/mL)	LH (mIU/mL)	FSH (mIU/mL)	TESTOSTERONE (ng/mL)
Control	0.47 ± 0.05^{b}	1.05 ± 0.25^{a}	4.53 ± 0.22^{b}	0.47 ± 0.05^{b}	1.05 ± 0.25^{b}	4.53 ± 0.22^{b}
Sham	0.47 ± 0.05^{b}	1.02 ± 0.19^{a}	4.10 ± 0.10^{b}	0.47 ± 0.05^{b}	1.02 ± 0.19^{b}	4.10 ± 0.10^{b}
I/R	0.16 ± 0.06^{a}	1.00 ± 0.18^{a}	3.63 ± 0.70^{a}	0.15 ± 0.05^{a}	0.90 ± 0.10^{a}	2.88 ± 0.71^{a}
I/R + 1000 mg/kg PP	0.52 ± 0.05^{b}	1.07 ± 0.24^{a}	4.56 ± 0.31^{b}	1.07 ± 0.09^{c}	1.10 ± 0.20^{b}	4.75 ± 0.25^{b}
I/R + 5000 mg/kg PP	0.80 ± 0.07^{c}	1.10 ± 0.18^{a}	4.59 ± 0.18^{b}	5.80 ± 0.09^{d}	1.15 ± 0.22^{b}	5.12 ± 0.08^{c}

Values are expressed as Mean ± SD of 6 determinations. I/R: Ischemia/Reperfusion; PP: Proxeed Plus; LH: Luteinizing hormone; FSH: Follicle Stimulating hormone, Sham: Rat underwent surgery without induction of torsion.

PP caused dose-dependent decreases of Caspase-3, Caspase-9, and iNOS levels when compared with the I/R group. However, at 7 days of treatment, no significant differences exist in Caspase-3 activity between PP treated groups, sham group and the control (Fig. 1). However, at 7 days of treatment, rats treated with 5000 mg/kg bw PP exhibited the least level of caspase-9 and iNOS when compared with the control and sham group (Fig. 1).

3.5. Histopathological and immunohistochemistry data

Histopathological evaluation of testicular tissue revealed severe germ cell loss, atrophy and disruption of the seminiferous epithelium in the I/R group. The Sham group shows normal tissue architecture (Plate 1 and 2). Rats treated with Proxeed Plus at 1000 and 5000 mg/kg bw showed improved testicular architecture than the I/R groups. The lowest spermatogenetic activity, reduced seminiferous tubular diameter and Leydig cell mass (Fig. 2) were found in the ischemia-reperfusion group. Groups that were administered Proxeed Plus had higher spermatogenetic activity scores than I/R animals. Group administered 5000 mg/kg bw Proxeed Plus had higher MSTD than the control. No significant diff ; erences in the Leydig cell mass were observed between the Control group, Sham group and PP treated groups. Analysis of the immunohistochemistry of the tissue samples showed that the iNOS expression was significantly higher in the I/R group while the control group and PP treated groups had lower (poor) iNOS expression (plate 3).

4. Discussion

Studies have implicated free radicals in ischemia-reperfusion induce testicular damage, apoptosis, and male infertility [19]. Therefore, effective antioxidants treatment is important in improving or salvaging a torsed testis. Oxidative stress arises from an imbalance between oxidants generation vs defense ability of intracellular antioxidants system [42]. It has been established that I/R induced oxidative stress can occur through the activity of non-radical oxidants like H_2O_2, macromolecular attacked by the oxidant radicals, or indirectly by nitrosative/oxidative modulation of regulatory proteins [43]. In the present study, the high oxidative stress status of I/R rats were indicated by the increase serum and tissue levels of MDA and H_2O_2 generations. Although the levels of superoxide anion radicals were not assayed in this study (O^{2-}) it is a highly reactive and pioneered free radical during I/R injury, and subsequently gives rise to H_2O_2 via dismutation [12]. The most important indicator of tissue injury due to I/R injury is the MDA level which is an indirect indicator of lipid peroxidation due to ROS effects [44]. The high levels of MDA in rats with I/R injury are indication of elevated ROS and oxidative stress. ROS cause chain reactions of lipid peroxidation in the cell membranes, which eventually leads to the generation of the major lipid peroxidation product, MDA [12] Fortunately, as a free radical scavenger agent and powerful antioxidant supplement Proxeed Plus exhibited a powerful effect on reducing MDA levels in the I/R treated rats.

GPx and SOD are major ROS scavenging enzymes in the reproductive system of male rats. Among the antioxidant enzymes, GPx constitutes the first line of defense against oxidative stress during ischemia-

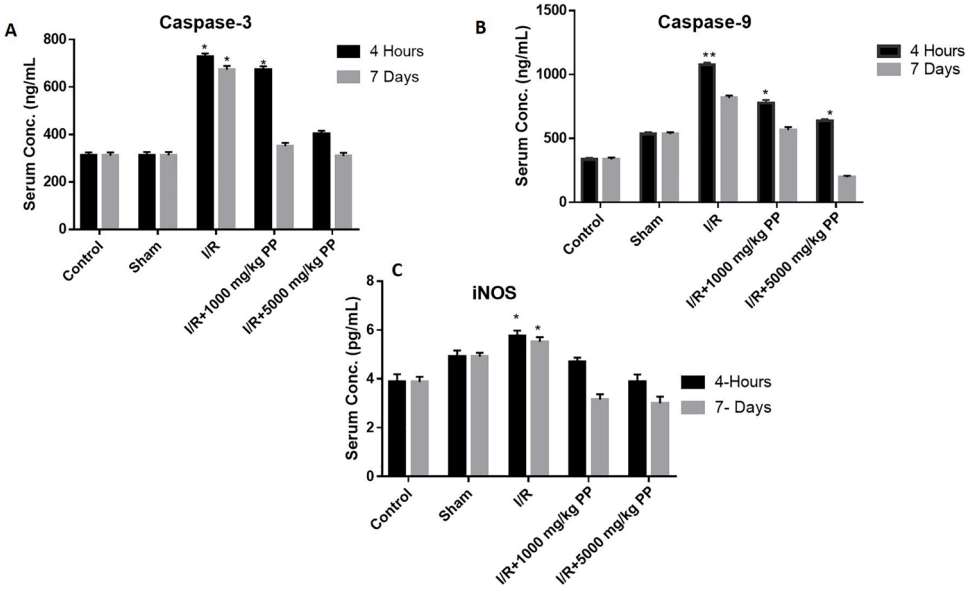

Fig. 1. Effects of Proxeed Plus on (a) Caspase-3 (b) Caspase-9 and (c) iNOS activities on ischemia−reperfusion injury in male rats. I/R: Ishemia/Reperfusion; PP: Proxeed Plus. Each bar represents Mean ± SD of 6 determinations. Bars with different superscript alphabets are significantly different ($p < 0.05$). *$p \leq 0.05$, **$p \leq 0.001$.

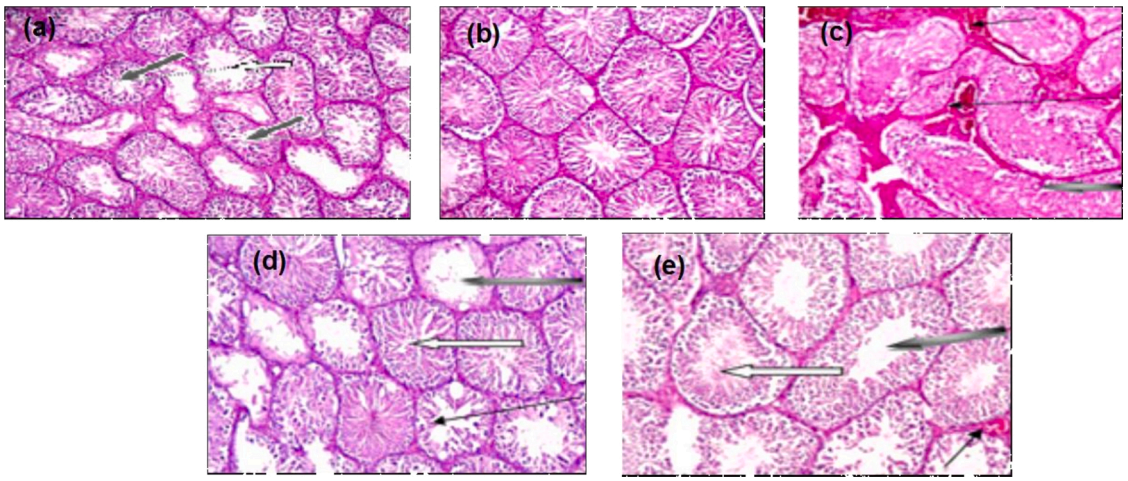

Plate 1. Photomicrograph of a rat's testicular section restrained by Haematoxylin and Eosin after 4 h treatment with PP. **(a)** CONTROL showing several normal testicular architectures, seminiferous tubules with normal germ cell layer and maturation stages. The interstitial spaces show normal Leydig cells, however, there are few seminiferous tubules with sloughed germ cells into their lumen and some with maturation arrest (black arrow). **(b)** SHAM showing normal testicular architectures, **(c)** I/R showing very poor architecture. with generalized and severe degeneration of seminiferous tubules, these tubules are fibrotic with thickened propria, moderately congested interstitial spaces (slender arrow), degenerated germinal epithelial cells and necrosis (black arrow), **(d)** I/R + PP (1000 mg/kg bw) showing normal germ cells maturation stages and spermatozoa, (white arrow).normal interstitial spaces (slender arrow) but there are seminiferous tubules with tubular vacuolation and thickened proppria **(e)** I/R + PP (5000 mg/kg bw) showing a moderately normal testicular architecture.

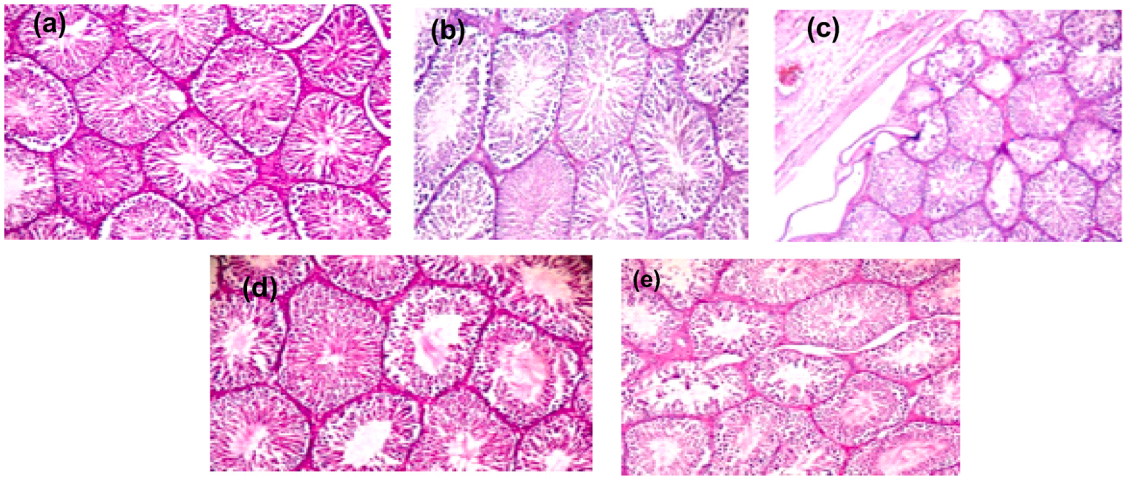

Plate 2. Photomicrograph of rat's testicular section stained by Haematoxylin and Eosin after 7 days treatment with PP. **(a)** CONTROL showing several normal testicular architectures, **(b)** SHAM showing normal testicular architecture, **(c)** I/R showing very poor testicular architecture. **(d)** I/R + PP (1000 mg/kg bw) showing moderately normal testicular architecture **(e)** I/P + PP (5000 mg/kg bw) showing normal testicular architecture.

reperfusion injury in testis [45]. However, the observed increased concentration of the toxic metabolic products (H_2O_2 and MDA) could result in decrease intracellular antioxidants defense system which in turn may cause loss of sperm motility and viability [46]. This is in line with the observed drastic reduction in the serum and epididymis activities of SOD, CAT, GST, GPx and GSH levels and increases levels of MDA observed in the I/R rats. Findings from previous studies have also shown increased MDA levels and antioxidant enzymes inactivation in rat testes following ischemia-reperfusion [27,47]. In agreement with the results of the present study, Ozbek et al. [48] and Ozturk et al. [49] also showed that testicular torsion for 4 h and detorsion increase tissue levels of MDA and reduce SOD and GPx levels.

The therapeutic effect of the supplement was evident by increase in SOD, CAT, GSH, GST, and GPx whereas a decrease in MDA levels in the treated rats. These findings agree with the study of Micic et al. [50] who reported that men treated with Proxeed Plus showed high sperm motility and seminal carnitine. Proxeed Plus has also been reported for a protective effect against toxicant-induced nephrotoxicity in experimental animal models [25]. The results of the present study are also supported by the study of Sugiyama et al. [51] who reported that a single dose of polyphenolic catechins given to a 4 h ischemia rats before reperfusion protected against testicular damage from I/R injury and inhibited a further decrease in the activity of SOD. Several experimental studies have also shown that I/R were considerably attenuated by treatment with SOD or SOD analogues [43,52]

LH, testosterone, and FSH play an important role in controlling testicular functions [53]. When LH is released by the pituitary gland, it acts upon the Leydig cells to produce testosterone [54]. Also, FSH plays an important function in spermatogenesis initiation and germ cell maturation. In the present study, I/R induced loss of hormonal activities as a significant reduction in the serum levels of LH, FSH, and testosterone were observed in I/R rats in comparison to the control, sham group and PP treated rats. However, the administration of Proxeed Plus caused a significant increase in FSH, LH, and testosterone when compared with the control and sham group, an indication that PP improves the quality of spermatogenesis and hormone against damage

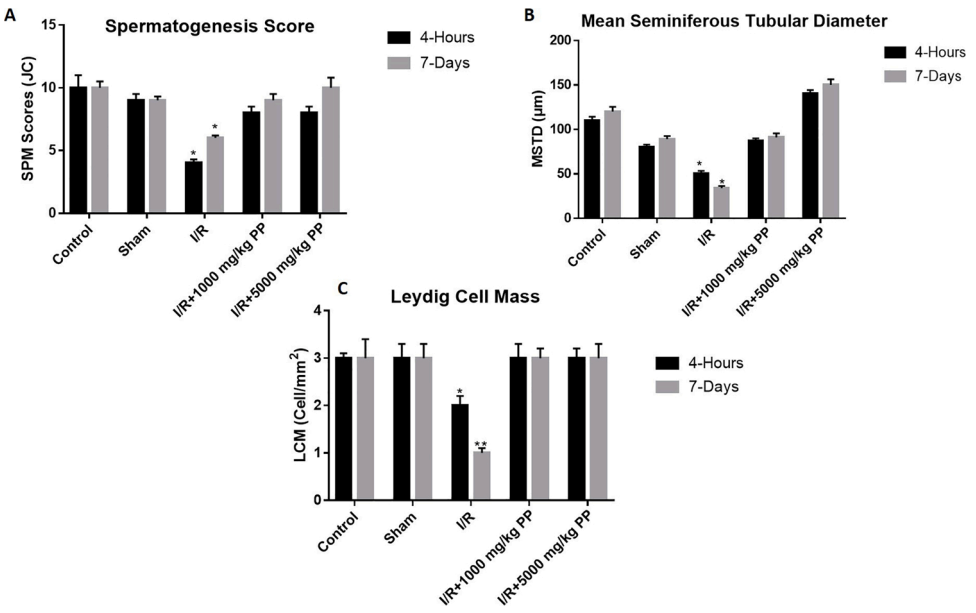

Fig. 2. Effects of Proxeed Plus on (a) (a) Spermatogenetic activity and not spermatogenesis activities (b) Mean seminiferous tubular diameter (MSTD) and (c) Leydig cell mass (LCM) of ischemia-reperfused testis after 4 h and 7 days treatment. I/R: Ishemia/Reperfusion; PP: Proxeed Plus. JC: Johnson's scores. Each bar represents Mean ± SD of 6 determinations. *p ≤ 0.05, **p ≤ 0.001.

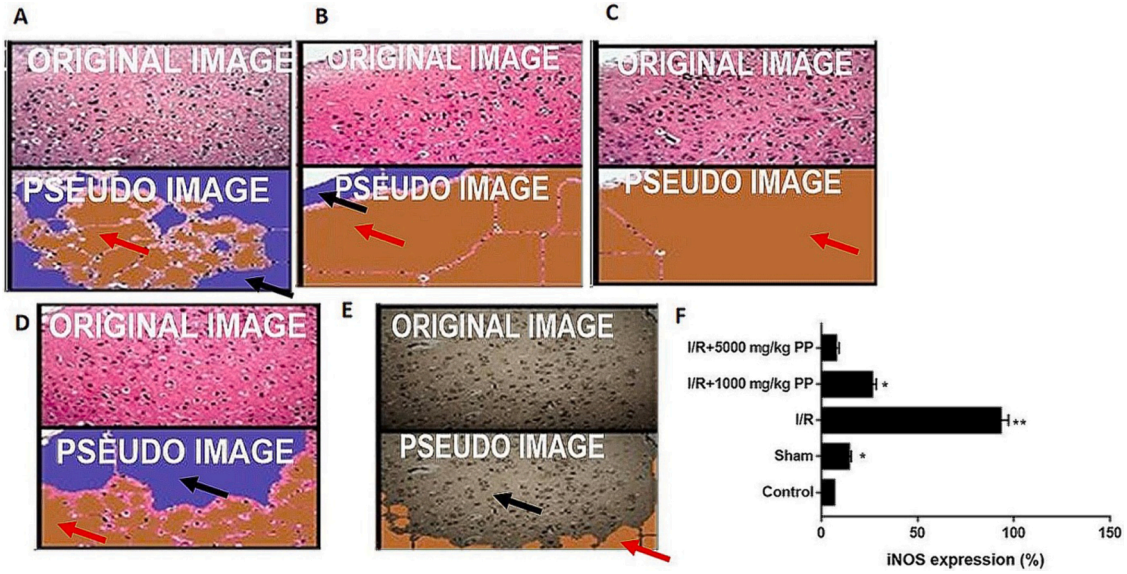

Plate 3. Representative immunohistochemistry images of inducible Nitric oxide synthase (iNOS) in proxeed plus treated ischemia-reperfusion injured rats. A: control (poor expression) B: Sham group (poor expression) C: I/R (strong expression) D: 1000 mg/kg Proxeed Plus + I/R (mild expression) E: 1000 mg/kg Proxeed Plus + I/R (poor expression). F: graph showing the % iNOS expression. I/R: Ishemia/Reperfusion; PP: Proxeed Plus Each bar represents Mean ± SD of 3 determinations. *p ≤ 0.05, **p ≤ 0.001. The Original image represents the photomicrograph of immune stained testicular cells showing the expression of inducible Nitric oxide while the pseudo colored image shows the percentage of positively stained nuclear area (labeling index) using colour deconvolution algorithms separating the counterstain. Brown colour: - the DAB precipitate indicate area of iNOS expression (red arrow), blue colour:- H & E counterstain indicate no expression of inducible nitric oxide synthase (negatively stained nuclear area of the testicular tissue) (black arrow).

caused by I/R of the testis.

Caspase 3 and caspase 9 are an apoptotic feature, thus the high levels of the caspase-3 and caspase-9 in I/R injured rats is an indication of the increase in programmed cell death following testicular I/R. Our data correspond with the results reported by other investigators, which demonstrated that testicular torsion induces germ cell-specific apoptosis [55,56]. Treatment with PP resulted in a significant decrease in the germ cell apoptosis in the ischemic testis.

The I/R injured rats exhibited significant higher iNOS immunohistochemical reactions. Our results agree with those of other studies that demonstrated an increase in iNOS expression as detected by immunohistochemistry in the organs of I/R injured rats [57]. The high expression of iNOS enhances the formation of NO and may contribute to circulatory failure and organ dysfunction. Besides, it has been reported that NO promotes apoptosis via activation of c-Jun [58,59]. These correspond with the high levels of apoptotic markers observed in I/R injured rats

Biochemical, histological and immunohistochemical evidence from this study have shown that I/R damage occurs following testis ischemia, which caused hormonal imbalance, apoptosis, spermatogenesis

impairment and may ultimately cause subfertility and infertility. Proxeed Plus has reversed this negative effect after a single administration and a more beneficial effect after repeated administrations. Clinical trials have shown that the ingredients in Proxeed Plus support sperm health, including increase sperm motility, count, speed, and concentration [50]. However, this study showed, for the first time that Proxeed Plus can ameliorate oxidative stress, apoptosis and hormonal impairment in ischemia-reperfused injured rats. Although the relevant mechanisms through which PP suppress the oxidative stress and iNOS remain a subject for future research, the present study indicates that PP could serve as a promising supplement to salvage ischemic testis and might further expand its therapeutic value in ischemic diseases.

5. Conclusions

The present study demonstrated that PP protected the rat's testis against I/R-induced oxidative stress, apoptosis and hormonal imbalance with maximum protective effect at 5000 mg/kg bw. This is suggestive of the unique and usefulness of PP, thus, it may be a novel approach in preventing testicular damage of reperfusion injury after ischemia. However, mechanistic experiments and clinical studies are necessary to confirm our findings.

Author contributions

J.O.S conceived the experiments, J.O.S and G.A designed the experiments, J.O.S, G.A and .Z.I performed the experiments, J.O.S and O. B.J analyzed the data, J.O.S and .Z.I treated the animals. B.L wrote the draft of the manuscript, J.O.S, O.B.J and F.M proof read the draft of the manuscript, B.L, J.O.S, O.B.J wrote the final manuscript. All authors read and approved the final manuscript

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

We appreciate the Department of Biochemistry, Bingham University for providing the laboratory facilities and technical support for the execution of this study. Also, authors appreciate the Institute of Chemical and Biotechnology, Faculty of Applied and Computer Science Research, Vaal University of Technology, South Africa, for supporting the research.

References

[1] H. Amani, et al., Would colloidal gold nanocarriers present an effective diagnosis or treatment for ischemic stroke? Int. J. Nanomedicine 14 (2019) 8013–8031.
[2] H. Amani, et al., Selenium nanoparticles for targeted stroke therapy through modulation of inflammatory and metabolic signaling, Sci. Rep. 9 (1) (2019) 6044.
[3] M. Ruiz-Meana, D. García-Dorado, Pathophysiology of ischemia-reperfusion injury: new therapeutic options for acute myocardial infarction, Rev. Espa. de Cardiol. 62 (2) (2009) 199–209.
[4] A. Panisello-Roselló, J. Roselló-Catafau, Molecular mechanisms and pathophysiology of ischemia-reperfusion injury, Int. J. Mol. Sci. 19 (12) (2018) 4093.
[5] M.Y. Wu, et al., Current mechanistic concepts in ischemia and reperfusion injury, Cell. Physiol. Biochem. 46 (4) (2018) 1650–1667.
[6] F.M. Ornellas, et al., Bone marrow-derived mononuclear cell therapy accelerates renal ischemia-reperfusion injury recovery by modulating inflammatory, antioxidant and apoptotic related molecules, Cell. Physiol. Biochem. 41 (5) (2017) 1736–1752.
[7] D.W. Filho, et al., Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia–reperfusion injury, Mol. Aspects Med. 25 (1) (2004) 199–210.
[8] J. Meštrović, et al., Protective effect of urapidil on testicular torsion-detorsion injury in rats, Surg. Today 47 (3) (2017) 393–398.
[9] Z. Pogorelić, et al., Testicular torsion in the inguinal canal in children, J. Pediatr. Urol. 9 (6 Pt A) (2013) 793–797.
[10] J. Meštrović, et al., Differentiation of inflammatory from non-inflammatory causes of acute scrotum using relatively simple laboratory tests: prospective study, J. Pediatr. Urol. 9 (3) (2013) 313–317.
[11] Z. Pogorelić, I. Mrklić, I. Jurić, Do not forget to include testicular torsion in differential diagnosis of lower acute abdominal pain in young males, J. Pediatr. Urol. 9 (6 Pt B) (2013) 1161–1165.
[12] G. Vaos, N. Zavras, Antioxidants in experimental ischemia-reperfusion injury of the testis: where are we heading towards? World J. Methodol. 7 (2) (2017) 37–45.
[13] D.N. Granger, Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury, Am. J. Physiol. 255 (6 Pt 2) (1988) H1269–75.
[14] M. Al-Maghrebi, W.M. Renno, Genistein alleviates testicular ischemia and reperfusion injury-induced spermatogenic damage and oxidative stress by suppressing abnormal testicular matrix metalloproteinase system via the Notch 2/Jagged 1/Hes-1 and caspase-8 pathways, J. Physiol. Pharmacol. 67 (1) (2016) 129–137.
[15] R. Dursun, et al., The protective effect of goji berry extract in ischemic reperfusion in testis torsion, Int. J. Clin. Exp. Med. 8 (2) (2015) 2727–2733.
[16] T. Hafez, et al., The effect of intraportal prostaglandin E1 on adhesion molecule expression, inflammatory modulator function, and histology in canine hepatic Ischemia/Reperfusion injury, J. Surg. Res. 138 (1) (2007) 88–99.
[17] M. de Perrot, et al., Prostaglandin E1 protects lung transplants from ischemia-reperfusion injury: a shift from pro- to anti-inflammatory cytokines, Transplantation 72 (9) (2001) 1505–1512.
[18] M. Kanter, Protective effects of Ginkgo biloba (EGb 761) on testicular torsion/detorsion-induced ischemia–reperfusion injury in rats, Exp. Mol. Pathol. 91 (3) (2011) 708–713.
[19] S. Arena, et al., Medical perspective in testicular ischemia-reperfusion injury, Exp. Ther. Med. 13 (5) (2017) 2115–2122.
[20] J. Bouayed, T. Bohn, Exogenous antioxidants–double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses, Oxid. Med. Cell. Longev. 3 (4) (2010) 228–237.
[21] G.M. Busetto, et al., Effect of metabolic and antioxidant supplementation on sperm parameters in oligo-astheno-teratozoospermia, with and without varicocele: a double-blind placebo-controlled study, Andrologia 50 (3) (2018).
[22] A. Agarwal, T.M. Said, Carnitines and male infertility, Reprod. Biomed. Online 8 (4) (2004) 376–384.
[23] X. Zhou, F. Liu, S. Zhai, Effect of L-carnitine and/or L-acetyl-carnitine in nutrition treatment for male infertility: a systematic review, Asia Pac. J. Clin. Nutr. 16 (Suppl 1) (2007) 383–390.
[24] M.K. Moslemi, S. Tavanbakhsh, Selenium-vitamin E supplementation in infertile men: effects on semen parameters and pregnancy rate, Int. J. Gen. Med. 4 (2011) 99–104.
[25] E.E. Dogan, et al., SP057EFFECTS of proxeed plus in the prevention and treatment of CISPLATIN NEPHROTOXICITY: an experimental rat study, Nephrol. Dial. Transplant. 33 (suppl_1) (2018) i364.
[26] F. Fırat, et al., Oxytocin for preventing injury due to testicular torsion/detorsion in rats, Ulus. Travma Acil Cerrahi Derg. 24 (2) (2018) 89–96.
[27] A. Gezici, et al., Effects of gabexate mesilate on ischemia-reperfusion-induced testicular injury in rats, Pediatr. Surg. Int. 22 (5) (2006) 435.
[28] O.K. Shittu, et al., Alteration in biochemical indices following chronic administration of methanolic extract of Nigeria bee propolis in Wistar rats, Asian Pac. J. Trop. Dis. 5 (8) (2015) 654–657.
[29] B. Lawal, et al., Antimicrobial evaluation, acute and sub-acute toxicity studies of Allium sativum, J. Acute Dis. 5 (4) (2016) 296–301.
[30] D.A. Adesina, et al., Antiplasmodial effect and sub-acute toxicity of alkaloid, flavonoid and phenolic extracts of Sida acuta leaf on Plasmodium berghei-infected animals, J. Taibah Univ. Sci. 14 (1) (2020) 943–953.
[31] L. Schwarzstein, et al., Luteinizing hormone (LH), follicle-stimulating hormone, and testosterone responses to consecutive injections of D-Leucine-6-LH-Releasing hormone ethylamide in normal men, Fertil. Steril. 28 (4) (1977) 451–455.
[32] Z.A. Placer, L.L. Cushman, B.C. Johnson, Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems, Anal. Biochem. 16 (2) (1966) 359–364.
[33] M.-L. Hu, 41] Measurement of protein thiol groups and glutathione in plasma. Methods in Enzymology, Academic Press, 1994, pp. 380–385.
[34] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem. 247 (10) (1972) 3170–3175.
[35] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1) (1959) 70–77.
[36] M.A. Koroliuk, et al., [A method of determining catalase activity], Lab. Delo (1) (1988) 16–19.
[37] A.G. Gornall, C.J. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (2) (1949) 751–766.
[38] P. Salgame, et al., An ELISA for detection of apoptosis, Nucleic Acids Res. 25 (3) (1997) 680–681.
[39] S.G. Johnsen, Testicular biopsy score count–a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males, Hormones 1 (1) (1970) 2–25.
[40] A. Al Drees, M. Salah Khalil, M. Soliman, Histological and immunohistochemical basis of the effect of Aminoguanidine on renal changes associated with hemorrhagic shock in a rat model, Acta Histochem. Cytochem. 50 (1) (2017) 11–19.
[41] E.C. Roosen-Runge, Quantitative investigations on human testicular biopsies: I. Normal testis, Fertil. Steril. 7 (3) (1956) 251–261.
[42] B. Lawal, et al., African natural products with potential antioxidants and hepatoprotectives properties: a review, Clin. Phytoscience 2 (1) (2016) 23.
[43] T. Kalogeris, et al., Cell biology of ischemia/reperfusion injury, Int. Rev. Cell Mol. Biol. 298 (2012) 229–317.

[44] A. Agarwal, R.A. Saleh, M.A. Bedaiwy, Role of reactive oxygen species in the pathophysiology of human reproduction, Fertil. Steril. 79 (4) (2003) 829–843.
[45] J.O. Olugbodi, et al., Silver nanoparticles stimulates spermatogenesis impairments and hematological alterations in testis and epididymis of male rats, Mol. (Basel, Switzerland) 25 (5) (2020) 1063.
[46] S. Dutta, A. Majzoub, A. Agarwal, Oxidative stress and sperm function: a systematic review on evaluation and management, Arab J. Urol. 17 (2) (2019) 87–97.
[47] A.H. Salmasi, et al., Effect of morphine on ischemia-reperfusion injury: experimental study in testicular torsion rat model, Urology 66 (6) (2005) 1338–1342.
[48] O. Ozbek, et al., The protective effect of apocynin on testicular ischemia-reperfusion injury, J. Urol. 193 (4) (2015) 1417–1422.
[49] H. Ozturk, et al., Interleukin 10 reduces testicular damage in experimental testicular ischemia/reperfusion injury, Urology 83 (2) (2014) 508, e1-6.
[50] S. Micic, et al., Oligoastenospermic men treated with proxeed plus showed correlation between sperm motility and seminal carnitine, Fertil. Steril. 106 (3) (2016) e298–e299.
[51] A. Sugiyama, et al., Beneficial effects of (−)-epigallocatechin gallate on ischemia-reperfusion testicular injury in rats, J. Pediatr. Surg. 47 (7) (2012) 1427–1432.
[52] B. Lima, et al., S-nitrosylation in cardiovascular signaling, Circ. Res. 106 (4) (2010) 633–646.
[53] S. Ramaswamy, G.F. Weinbauer, Endocrine control of spermatogenesis: role of FSH and LH/ testosterone, Spermatogenesis 4 (2) (2015) e996025.
[54] T.R. Kumar, et al., Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility, Nat. Genet. 15 (2) (1997) 201–204.
[55] T.T. Turner, Acute experimental testicular torsion. No effect on the contralateral testis, J. Androl. 6 (1) (1985) 65–72.
[56] T.T. Turner, et al., Acute testicular ischemia results in germ cell-specific apoptosis in the rat, Biol. Reprod. 57 (6) (1997) 1267–1274.
[57] C. Thiemermann, et al., Vascular hyporeactivity to vasoconstrictor agents and hemodynamic decompensation in hemorrhagic shock is mediated by nitric oxide, Proc. Natl. Acad. Sci. U. S. A. 90 (1) (1993) 267–271.
[58] M. Amir, et al., Distinct functions of JNK and c-Jun in oxidant-induced hepatocyte death, J. Cell. Biochem. 113 (10) (2012) 3254–3265.
[59] M. Yamaguchi, et al., An immunohistochemical study of the localization of inducible nitric oxide synthase (iNOS) and heat shock protein (HSP) in Pleomorphic Adenoma, Acta Histochem. Cytochem. 37 (4) (2004) 267–271.