normalized NO levels. CH pretreatment prevented the PQ-mediated depletion of glutathione (GSH), a critical antioxidant. These findings demonstrate that chrysin effectively protects against PQ-induced testicular oxidative stress by improving antioxidant defense, suggesting its potential to safeguard against male reproductive dysfunction.

Keywords: Paraquat, herbicide, testicules; toxicity, stress, chrysin

INTRODUCTION

Maintaining male fertility is crucial for healthy reproduction and population sustainability. Paraquat (PQ), a widely used herbicide in agriculture, poses significant environmental and health risks, serving both as a herbicide and an environmental hazard threat (Richardson *et al*., 2019; El-Saber Batiha *et al*., 2020). PQ primarily targets lung alveolar cells, leading to conditions such as lung fibrosis and fatal asphyxia (Shabrina *et al*., 2023; Tambuzz *et al*., 2024). In addition to its pulmonary effects, PQ poisoning in rats has been linked to neurological degenerative conditions resembling Parkinson's disease, underscoring its systemic toxicity (Wu *et al*., 2022; Ibarra-Gutiérrez *et al*., 2023). The acute and prolonged exposure to PQ provokes oxidative stress, disrupting the delicate balance of the redox system (Jafari *et al*., 2021; Wu *et al*., 2022; Ibarra-Gutiérrez, *et al*., 2023). This oxidative burden can manifest in various tissues, including the testes, where high levels of free radicals can lead to peroxidative damage, akin to that seen in testicular injury in models of torsion/detorsion (Minas *et al*., 2023). The testes are highly susceptible to oxidative damage due to their high metabolic activity and lipid-rich composition. Reactive oxygen species generated by PQ can induce lipid peroxidation of membranes and damage to DNA, proteins and organelles in testicular tissues. This oxidative stress has been shown to impair spermatogenesis at multiple stages, reducing sperm quality and quantity, and decreasing fertility (Zhao *et al*., 2021).

Antioxidant supplementation has shown promise in mitigating such oxidative damage associated with testicular injuries (Mohamed *et al*., 2021). In this context, CH, a flavonoid found in propolis, pollen, honey and certain plants has garnered attention for its potential protective effects on testicular function. Chrysin has been reported to modulate testosterone levels by inhibiting the enzymes involved in its conversion to estradiol, while also exhibiting anti-inflammatory properties (Vazhacharickal, 2021; Banihani, 2022; Shahbaz *et al*., 2023). Moreover, CH has shown antiapoptotic effects, reducing germ cell apoptosis through various mechanisms, including the regulation of testis apoptotic proteins (Abadi *et al*., 2023; Şimşek *et al*., 2023).

Despite the known benefits of CH for testicular health, research on its application in mitigating PQ-induced testicular dysfunction is scarce. Exposure to paraquat remains a health concern, and there is a need for effective strategies to protect male reproductive function. Elucidating whether CH pretreatment can attenuate PQ-induced oxidative stress and preserve male reproductive function is therefore highly warranted. The current study aimed to address this knowledge gap and evaluate the protective potential of CH against PQ-induced testicular oxidative stress in Wistar rats. It was hypothesized that CH pretreatment would alleviate PQ-mediated

oxidative stress and safeguard against potential reproductive dysfunction. This research is intended to contribute to knowledge on mitigating PQ-induced reproductive toxicity and explore the potential therapeutic application of CH in this context.

MATERIALS AND METHODS

Chemicals

Paraquat dichloride (PQ; C12H14Cl2N2, CAS No. 1910-42-5) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chrysin (5,7-dihydroxyflavone; C15H10O4, CAS No. 480-40-0) was purchased from Solarbio Science and Technology (Beijing, China). Other reagents included 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), aniline-p-sulfonic acid, monophosphoric acid, 2-(1-naphthylamino) ethylamine dihydrochloride, trometamol, hydrochloric acid, pyrogallol, diammonium molybdate, sodium azide, monopotassium phosphate, dipotassium phosphate, and dihydro-2-thioxo-4,6 (1H,5H)-pyrimidinedione. All chemicals were of high purity grade suitable for biochemical analysis and were obtained from either Sigma-Aldrich or Solarbio, unless otherwise stated. Deionized water was used to prepare all reagents and solutions.

Animals, grouping and dosing

Twenty-eight male Wistar albino rats (10–12 weeks old, 200–250 g) were obtained from the Department of Biochemistry, Federal University of Agriculture Abeokuta, Nigeria. The study was approved by the FUNAAB Research Ethical Committee (approval number: FUNAAB/COLBIOS/PG/15-0171) and adhered to the ARRIVE guidelines (Percie du Sert *et al.*, 2020). Animals were housed under controlled conditions (24 ± 1°C temperature, 45 ± 5% humidity, and 12-hour light/dark cycle) with *ad libitum* access to standard rodent chow and water. One week of acclimatization was observed before the dosing.

Rats were randomly assigned to four groups (n = 7/group): control (NC), chrysin (CH), paraquat (PQ), and chrysin + paraquat (CH+PQ). The NC and PQ groups received daily oral gavage of olive oil (vehicle) for seven days. The CH and CH+PQ groups received daily oral gavage of chrysin (100 mg/kg body weight) dissolved in olive oil for seven days. On the eighth day, the PQ and CH+PQ groups received a single PQ (35 mg/kg body weight, p.o.) administration.

Administration took place daily from 7:30 to 8:30 AM via oral gavage. The justification for the sub-acute dose schedule (consisting of one week of chrysin pretreatment intervention followed by a single administration of PQ) is consistent with previous research on chrysin (Belhan *et al.*, 2019) and PQ (Mirzaee *et al.*, 2019). The dosage of chrysin (100 mg/kg) was chosen based on its well-documented antioxidant benefits, as indicated in the research by Belhan *et al.* (2019). The dosage of paraquat used (35 mg/kg) corresponds to 10% of the oral LD^{50} published in the report by WHO (2007).

Testicular tissue homogenization for oxidative stress and antioxidant assays

Testicular homogenates were prepared for subsequent analysis of oxidative stress and antioxidant markers. Twenty-four hours after PQ administration, rats were euthanized, and testes were collected. Following excision, each testis was decapsulated and rinsed with ice-cold saline (0.9% NaCl) to remove blood. A weighed portion (0.4 g) of testicular tissue

was homogenized in 3.6 mL of 0.1 M phosphate buffer (pH 7.4) using a Teflon homogenizer to achieve a 10% (w/v) homogenate. The homogenate was then centrifuged at 4,000 rpm for 10 minutes at 4°C. The resulting supernatants were aliquoted and stored at -20°C for subsequent analyses.

Testicular homogenates were prepared for oxidative stress and antioxidant marker analysis, with minor modifications to the method of Kurien *et al.* (2004). Twenty-four hours after PQ administration, rats were euthanized, and testes were collected. Following excision, each testis was decapsulated and rinsed with ice-cold saline (0.9% NaCl) to remove blood. A weighed portion (0.4 g) of testicular tissue was homogenized in 3.6 mL of 0.1 M phosphate buffer (pH 7.4) using a Teflon homogenizer to achieve a 10% (w/v) homogenate. The homogenate was then centrifuged at 4,000 rpm for 10 minutes at 4°C. The resulting supernatants were aliquoted and stored at -20°C for subsequent analyses.

Oxidative stress and antioxidant assays

The oxidative stress and antioxidant markers were determined in the tissues as follows:

Nitric oxide (NO) assay

Testicular tissue NO levels were determined using the Griess method as described by (Sreejayan and Rao, 1997). This method utilizes the conversion of nitrite (NO2-) and nitrate (NO3-) to a colored azo compound under acidic conditions. Briefly, 1 mL of homogenate was incubated with an equal volume of 1% sulfanilamide in 5% phosphoric acid for 15 minutes in the dark. Subsequently, 0.1% N-(1-naphthyl) ethylenediamine (NED) was added (1 mL), followed by thorough mixing and a further 10-minute incubation at room temperature in the dark. Absorbance was measured at 520 nm within 15 minutes. A standard curve generated with sodium nitrite (NaNO2) was used to quantify NO levels in the samples. NO concentration (mol/L) was calculated as y - 0.03955)/0.1237, where y represents the sample absorbance, followed by calculations of NO concentration in g/g tissue.

Reduced glutathione (GSH) assay

Reduced glutathione (GSH) concentration, a key antioxidant, was determined in testes tissue using the Ellman method (1959). This spectrophotometric assay quantifies the formation of a yellow complex between Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid), DTNB] and the thiol group of GSH. The absorbance is measured at 412 nm. Briefly, 25 µL of testes tissue was mixed with 25 µL of 10% trichloroacetic acid (TCA) and centrifuged at 4,000 rpm for 5 minutes. Subsequently, 34 µL of the supernatant was transferred to a new tube and incubated with 350 µL of freshly prepared GSH working reagent (68.63 mL GSH buffer and 1.37 mL of 1 mM DTNB solution) for 10 minutes at room temperature. Absorbance was measured at 412 nm against a blank. GSH concentration was initially calculated as mol/L followed by g/g tissues.

$$\text{GSH conc. (mol/L)} = \frac{(\text{abs of sample} - \text{abs of blank})}{\text{b x } \varepsilon}$$

Where 14.150 $M^{-1}cm^{-1}$ is the molar extinction coefficient (ε) of the DTNB-GSH complex and the cuvette length (b) is typically 1 cm.

Glutathione-S-transferase (GST) assay

GST activity was assessed using a modification of the method described by Habig *et al.* (1974). This assay exploits the high GST isozyme activity with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. GST -mediated conjugation of CDNB with reduced glutathione results in a shift in its peak absorbance to a longer wavelength. The increase in absorbance at 340 nm, monitored over time, reflects the rate of the enzymatic reaction. Briefly, reaction mixtures were prepared in cuvettes labeled "blank" and "test." Each cuvette contained 0.15 mL CDNB, 0.03 mL reduced glutathione, and 2.82 mL phosphate buffer [0.1 M, pH 7.4, (blank)] or 2.79 mL phosphate buffer and 0.03 mL sample (test). The reaction was initiated by adding the sample to the test cuvette, and the absorbance at 340 nm (yellow chromophore of CDNB-SG conjugate) was measured against the blank every 60 seconds for 2 minutes.

$$\text{GST specific activity (µmole of CDNB} - \text{SG conjugate/min)/mg protein} = \frac{\frac{\Delta A340}{\text{min}} \times \text{reaction volume} \times \text{dilution factor}}{\text{b x } \varepsilon \times \text{sample volume} \times \text{mg protein/mL}}$$

The molar extinction coefficient (ε) at 340 nm = 9.6 mM^{-1} cm^{-1} and the cuvette length (b) is typically 1 cm.

Superoxide dismutase (SOD) assay

Superoxide dismutase activity in testicular homogenates was assessed using the Marklund and Marklund (1974) method. This technique measures the inhibition of pyrogallol auto-oxidation by SOD, leading to a decrease in absorbance at 420 nm. Briefly, 20 µL of sample (distilled water for blank) was mixed with 180 µL of 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM EDTA. After a 10-minute incubation, the reaction was initiated by adding 50 µL of 1 mM pyrogallol solution. Changes in absorbance at 420 nm were recorded every 30 seconds for 3 minutes to monitor the rate of pyrogallol auto-oxidation inhibition by SOD. The SOD activity was calculated and expressed as units per milliliter.

$$\text{inhibition} = \left(\frac{\text{abs/ min of blank} - \text{abs/ min of sample}}{\frac{\text{abs}}{\text{min}} \text{of blank}} \right)$$

$$\text{SOD unit activity } \left(\frac{\text{U}}{\text{mL}}\right) = \left(\frac{\text{\% inhibition}}{0.5}\right) \times \left(\frac{\text{Vt}}{\text{Vs}}\right) \times \text{df}$$

Where, Vt, Vs and df are total volume, sample volume and dilution factor, respectively

Catalase assay

Catalase activity in testicular homogenates was determined using the method by Shangari and O'Brien (2006). This assay measures the H2O2-consuming activity of catalase by monitoring the remaining H2O2 via its reaction with ammonium molybdate to form a yellow molybdate-H2O2 complex with a peak absorbance at 405 nm. Briefly, 0.2 mL of sample was mixed with 1.0 mL of

substrate solution (65 µmol/L H_2O_2 in 60 mM sodium-potassium phosphate buffer, pH 7.4) and incubated at 37°C for 60 seconds. Control reactions included a blank (1 mL H_2O_2 and 0.2 mL phosphate buffer) and a buffer control (1.2 mL phosphate buffer without enzyme or substrate). The reaction was terminated by adding 1.0 mL of 32.4 mM ammonium molybdate solution, and the absorbance at 405 nm was measured against the blank.

$$\text{Catalase unit activity } \left(\frac{\text{KU}}{\text{L}}\right) = \left(\frac{\text{abs of sample} - \text{abs of blank})}{\text{abs of standard} - \text{abs of blank}}\right) \text{ x } 271$$

Where 271 depicts a factor derived by Goth (1991).

Glutathione peroxidase (GPx) assay

GPx activity in testicular homogenates was appraised using the Rotruck *et al.* (1973) method. This assay indirectly quantifies GPx activity by measuring the remaining GSH after its consumption by GPx to reduce H_2O_2. Briefly, 25 µL of sample was incubated with 75 µL of GPx working reagent (containing 2.5 mM H_2O_2, 4 mM GSH, 10 mM NaN_3, and 0.4 M phosphate buffer, pH 6.5) at room temperature for 5 minutes. The reaction was terminated with 25 µL of 10% TCA and centrifuged at 4,000 rpm for 5 minutes. Subsequently, 34 µL of the supernatant was mixed with 350 µL of GSH working reagent (containing 0.1 M phosphate buffer containing 1 mM EDTA, pH 7.8 and 10 mM DTNB) and incubated for 10 minutes at room temperature. The formation of a yellow complex between GSH and DTNB was measured by absorbance at 412 nm.

$$\text{GSH consumed } \left(\frac{\text{mol}}{\text{L}}\right) = \left(\frac{\text{abs of sample} - \text{abs of blank}}{\text{b x } \varepsilon}\right)$$

Where 14.150 $M^{-1}cm^{-1}$ is the molar extinction coefficient (Ɛ) of the DTNB-GSH complex and the cuvette length (b) is typically 1 cm.

$$\text{GPx unit activity } \left(\frac{\text{U}}{\text{mL}}\right) = \left(\frac{\text{GSH consumed } \left(\frac{\text{mol}}{\text{L}}\right)}{10}\right)$$

Where 10 represents the time of incubation in minutes.

Malondialdehyde (MDA) assay

Malondialdehyde concentration, a marker of lipid peroxidation, was quantified in testicular tissue homogenates using the thiobarbituric acid reactive substances (TBARS) assay based on Fermandex *et al* (1997). This method leverages the reaction between TBA and MDA, a byproduct of lipid peroxidation, to form a pink chromophore with peak absorbance at 532 nm under acidic and high-temperature conditions. Briefly, 1.0 mL samples or blanks (distilled water) were mixed with 2.0 mL TBARS reagent (15% trichloroacetic acid, 0.37% TBA, and 0.25 M HCl in a 1:1:1 ratio). Butylated hydoxytoluene [0.1 mL of 0.02% w/v)] which completely prevents the formation of any non-

specific TBARS, was added. The mixture was incubated in a boiling water bath for 15 minutes, followed by immediate ice-cooling and centrifugation at 3,500 rpm for 10 minutes. The supernatant absorbance was measured at 532 nm using a blank for reference. MDA concentration (M) was calculated as absorbance divided by the molar extinction coefficient (ε) of the MDA-TBA complex (ε = 1.56 x 10^5 M^{-1} cm^{-1} at 535 nm). This was followed by calculations of MDA concentration in g/g tissue.

$$\text{MDA Conc. (mol/L)} = \frac{\text{(abs of sample)}}{\text{b x } \varepsilon}$$

Statistical analysis

Quantitative data is displayed as the mean value plus or minus the standard error of the mean (SEM). The group differences were evaluated using a one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. A significance level of $p < 0.05$ was deemed to have statistical significance. The Statistical Package for Social Sciences (SPSS) version 20.0 was used for statistical analyses, while GraphPad Prism version 8.0 was used to create graphical representations of the data.

RESULTS

Increased nitric oxide levels ameliorated by chrysin

Paraquat exposure significantly elevated NO levels by 72.73% compared to the control group (Figure 1). However, chrysin pretreatment significantly reduced this increase to 42.37%. Notably, chrysin alone did not significantly alter NO levels in healthy rats.

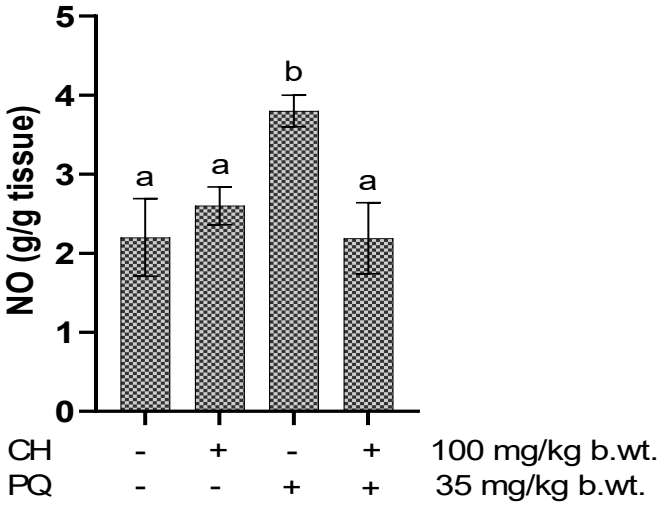

Figure 1: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced increase in the level of nitric oxide in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a or b) indicate a significant difference at $P < 0.05$.

Reduced glutathione levels are partially protected by chrysin.

Rats challenged with PQ resulted in a significant decreased in GSH levels by 7.18% over control (Figure 2). Chrysin pretreatment attenuated this reduction, the effect was not statistically significant. Chrysin alone did not affect GSH levels in healthy rats (Figure 2).

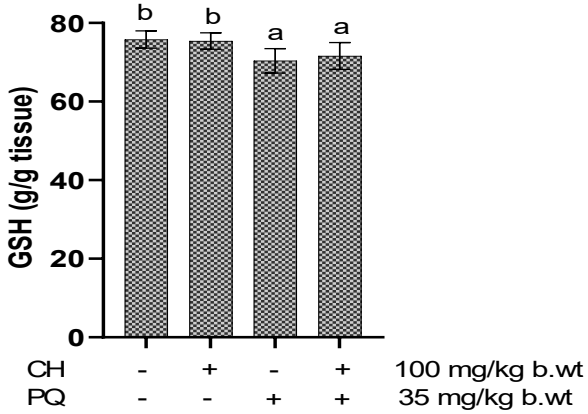

Figure 2: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced decrease in the level of glutathione in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a or b) indicate a significant difference at $P < 0.05$.

Chrysin significantly protects glutathione S-transferase activity.

PQ administration evoked a substantial declined in GST activity by 85.77% versus the standard control (Figure 3). Conversely, chrysin pretreatment significantly mitigated this decrease by 321.05%. Chrysin alone reduced GST activity compared to the control group (Figure 3).

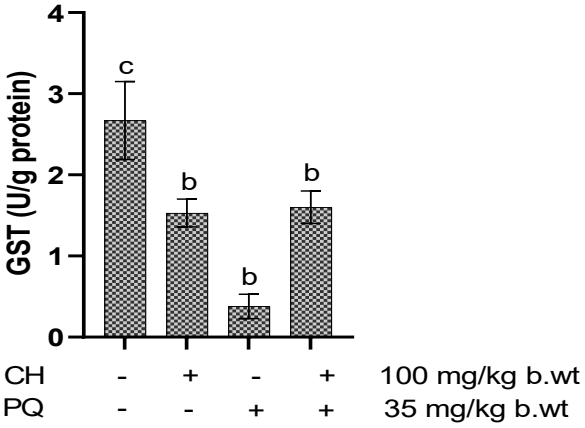

Figure 3: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced decrease in the activity of glutathione S-transferase in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a, b, c) indicate a significant difference at $P < 0.05$.

Chrysin protects superoxide dismutase activity.

PQ-treated rats elicited a significantly decreased SOD activity by 69.58%. However, chrysin pretreatment significantly reversed this reduction by 54.92%. Chrysin alone also caused a slight decrease in SOD activity compared to the control group, although this effect was not statistically significant (Figure 4).

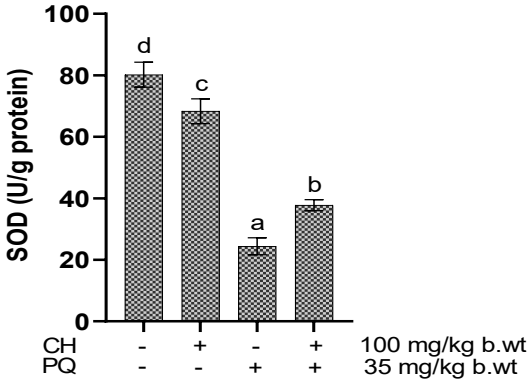

Figure 4: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced decrease in the activity of superoxide dismutase in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a, b, c) indicate a significant difference at $P < 0.05$.

Chrysin safeguards catalase activity.

PQ exposure significantly reduced catalase activity by 57.45% over control. Contrariwise, chrysin pretreatment significantly reversed this PQ-induced decline to the tune of 92.72%. Chrysin alone did not statistically affect catalase activity in healthy rats (Figure 5).

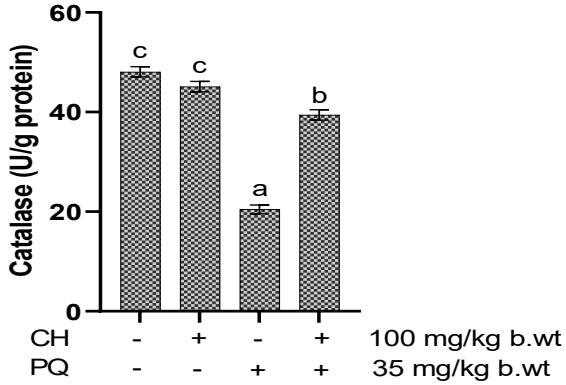

Figure 5: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced decrease in the activity of catalase in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a, b, c) indicate a significant difference at $P < 0.05$.

Chrysin offers partial protection to glutathione peroxidase activity.

Rats challenged with PQ triggered a significant decrease of 12.99% in GPx activity compared with the standard control group (Figure 6). Chrysin pretreatment restored this reduction. Chrysin alone significantly increased (10.36%) GPx activity compared to the control group (Figure 6).

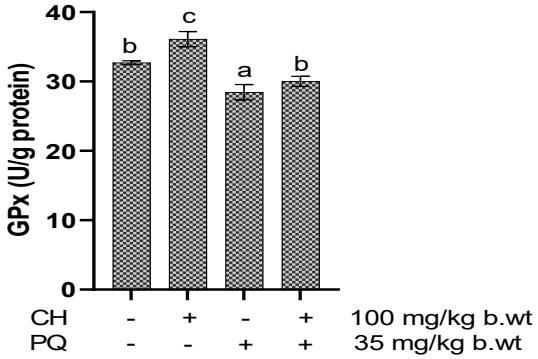

Figure 6: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced decrease in the activity of catalase in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a, b, c) indicate a significant difference at $P < 0.05$.

Chrysin protects glutathione reductase activity.

PQ exposure caused a significant decrease in GR activity by 59.63%. Chrysin pretreatment however attenuated this decline by 28.11% (Figure 7). Chrysin alone also caused a significant decrease in GR activity compared to the control group (Figure 7).

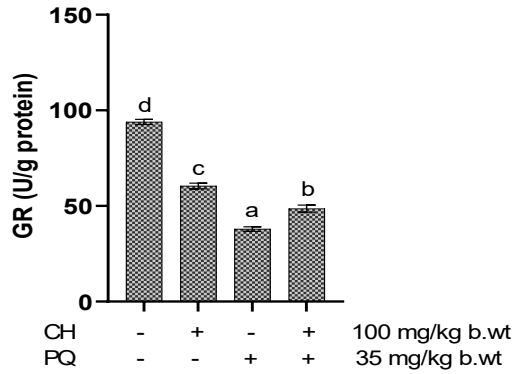

Figure 7: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced decrease in the activity of glutathione reductase in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a, b, c) indicate a significant difference at $P < 0.05$.

Chrysin reduces malondialdehyde levels.

PQ exposure significantly increased MDA concentration, a marker of lipid peroxidation, by 70.73%. Nonetheless, chrysin pretreatment reduced this elevation by 17.14%. Chrysin alone did not affect MDA levels compared to the control group (Figure 8).

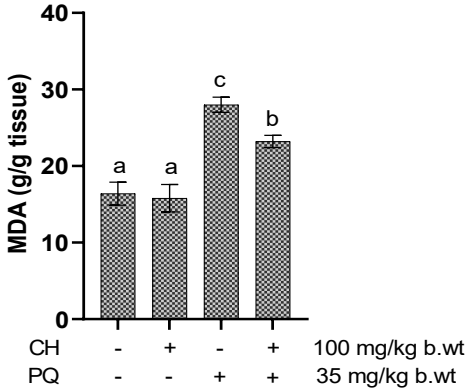

Figure 8: Effects of chrysin (CH) pretreatment on paraquat (PQ)-induced increase in the level of malondialdehyde in testis of rats. Control (2 mL/kg body weight olive oil), CH (100 mg/kg body weight) only, PQ (35 mg/kg body weight) only, CH (100 mg/kg body weight) + PQ (35 mg/kg body weight). Bars displaying different letter designations (a, b, c) indicate a significant difference at $P < 0.05$.

DISCUSSION

This exploration examined the protective impacts of oral CH pretreatment on paraquat (PQ)-mediated testicular oxidative stress in a male Wistar albino rat model. Data showed that oral paraquat exposure significantly spurred increased testicular nitric oxide (NO) levels and elevated malondialdehyde (MDA) contents while concomitantly diminishing the non-enzymatic reduced glutathione and antioxidant enzymes, including glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). Notably, pretreatment with chrysin effectively abated these paraquat-incited perturbations, restoring most parameters to control or near-control levels.

Exposure to paraquat (PQ), a widely used herbicide, is well-documented to generate excessive reactive oxygen species (ROS) such as superoxide radicals (O2*-), hydrogen peroxide (H2O2), and hydroxyl radicals (OH•) - Jafari *et al.*, 2021; Wu *et al.*, 2022; Ibarra-Gutiérrez *et al.*, 2023. These ROS can oxidize cellular macromolecules like proteins, lipids and DNA, disrupt antioxidant enzyme activities, and induce oxidative stress in various

tissues, including testes (Ijaz *et al*., 2022), leading to cytotoxicity, cell death, and various tissue dysfunctions (Ijaz *et al*., 2022), including male reproductive tract impairment, decreased sex hormone production, and germ cell loss (Cheng *et al*., 2017; Zhao *et al*., 2021, Minas *et al*., 2023; Shabrina *et al*., 2023).

Research on chrysin, a flavonoid compound, suggests its protective properties against oxidative stress in various models (Vazhacharickal, 2021; Banihani, 2022; Shahbaz *et al*., 2023). The specific molecular targets and signaling pathways involved in chrysin's protective effects against PQ-evoked testicular oxidative stress and cytotoxicity might be eclectic and involve its influence on various antioxidant pathways:

This current investigation showed that the use of chrysin pretreatment resulted in a reduction of the PQ-elicited elevated levels of testicular nitric oxide (NO). NO is a crucial physiological signaling inorganic gas molecule, exhibiting widespread distribution throughout many cells, tissues and organs. It affects cells generating it and those nearby, affecting several physiological and pathological processes (Luo *et al*., 2021). It plays multifaceted roles in male and female reproductive physiology, impacting various aspects such as neuromodulation, follicular and oocyte maturation, ovulation, corpus luteum degeneration, fertilization, implantation, pregnancy maintenance, labor and menstrual cycle regulation, spermatogenesis, sperm maturation and capacitation (Herrero *et al*., 2023; Luo *et al*., 2021). High or low NO contents prompt pathological consequences. NO deficit is remedied by supplementation, while excessive NO is cytotoxic. Elevated NO combines with superoxide radicals (O2*-) to generate nitrogen III oxide (N2O3) and decidedly reactive peroxynitrite anion (ONOO-), endorsing oxidative stress signature (Mustafa *et al*., 2009). Peroxynitrite anion, a strong oxidant that oxidizes protein sulfhydryl groups, iron-sulfur centers, zinc finger structures, nitrifies protein tyrosine residues, inactivates many essential proteins and enzymes, affects cell metabolism, inhibits respiratory chain enzymes, destroys mitochondrial structure, breaks DNA, and initiates lipid peroxidation, causing tissue damage (Dawson & Dawson, 2018). Chrysin's capability to regulate NO levels might implicate modulation of its generation or scavenging pathways and hence could act as a therapeutic potential of targeting NO pathways for managing reproductive health conditions.

GSH, a tripeptide composed of glutamate, cysteine, and glycine, is the most prevalent low-molecular-weight thiol molecule in all eukaryotic cells and is exclusively synthesized in the cytosol. GSH protects cells from oxidative damage and xenobiotic electrophile toxicity and maintains redox equilibrium by reducing the oxidants and conjugating the electrophiles (Vašková *et al*., 2023). In scavenging PQ-triggered free radicals (Wu *et al*., 2022), GSH levels may decline; hence, oxidative stress depletes GSH. Similar to previous studies (Xiang *et al*., 2022; Gonzales-Moreno *et al*., 2023), PQ-treated rats had significantly lower jejunum; also ileum of paraquat-treated piglets GSH levels than controls. Chrysin pretreatment restores the PQ-evoked GSH declines, possibly by increasing the GSH biosynthesis or stabilizing the oxidants via the electron/hydrogen donating mechanism. So, chrysin supplementation may serve as a therapeutic approach for manipulating GSH content in PQ poisoning and PQ-evoked reproductive health issues.

GST is a family of phase II biotransformation enzymes that detoxify xenobiotics and various reactive electrophiles, commonly produced by cytochrome P450 metabolism by conjugating (nucleophilic addition and substitution reactions) them with GSH, facilitating their elimination. GSTs are ubiquitous, although tissue-specific distribution may be an adaptive response to endo- and exogenous metabolites (Xiang *et al*., 2022). GSTs have membrane-bound microsomal and soluble cytosolic superfamilies. In PQ-mediated toxicosis, GSTs might explicitly target PQ or its metabolites for detoxification. The significant preservation of GST activity by chrysin indicates its role in bolstering the cell's capacity to eliminate paraquat-induced toxicants.

SOD is a group of metalloenzymes, including Cu, Zn-SOD, Mn-SOD, Fe-SOD and Ni-SOD, that are present but unequally distributed in all biological kingdoms and subcellular compartments (Zheng *et al*., 2023). Along with GSH, SOD also serves as the first line of defense against endogenous xenobiotics, exogenous xenobiotics, and reactive oxidants-engendered oxidative damage leading to various disorders and diseases (Juan *et al*., 2021). SOD dismutates relatively toxic superoxide anion free radical (O2*-) into molecular oxygen and hydrogen peroxide (Zheng *et al*., 2023). The observed chrysin's protection of SOD activity is vital as O2*- can occasion the generation of even more destructive ROS via the Haber-Weiss reaction (Juan *et al*., 2021).

Catalase decomposes H2O2 into water and oxygen molecules. Preserving catalase activity is essential as H2O2 can participate in the Fenton reaction (Juan *et al*., 2021), forming highly damaging hydroxyl radicals. Catalase deficiency or dysfunction has been implicated in various age-related degenerative disorders, including diabetes, hypertension, anemia, vitiligo, Alzheimer's, Parkinson's, bipolar disorder, cancer, and schizophrenia (Nandi *et al*., 2019). Thus, numerous laboratories are investigating its usage as a medication to treat such conditions. The observed remarkable restoration of catalase activity by chrysin highlights its role in preventing the Fenton detrimental pathway.

GPx, a family (GPx1-GPx8) of antioxidant selenoenzymes and heme-free thiol peroxidases that employ GSH as a cofactor/reducing agent to reduce H2O2 and organic hydroperoxides to water or corresponding alcohols, respectively thus combating their cytotoxicity, mitigating oxidative stress and upholding redox homeostasis (Pei *et al*., 2023). In addition to H2O2 detoxification, GPX4 is the only GPX family capable of reducing and destroying complex lipid (phospholipids, cholesterol, and cholesterol esters) hydroperoxides. GPX5, expressed chiefly in epididymal tissue, protects sperm against oxidative damage (Pei *et al*., 2023). The observed GPx could be a result of decreased GSH contents. We elucidated that chrysin partially protected GPx activity, signifying it may stimulate specific pathways within the antioxidant defense network.

In stabilizing and detoxifying oxidants, GSH is converted to oxidized glutathione (GSSG). GSH is regenerated from GSSG via NADPH-dependent glutathione reductase. The GSH/GSSG and NADPH/NADP+ are the most critical redox couples in sustaining cellular redox homeostasis (Vašková *et al*., 2023). GR is a homodimeric flavoprotein and the second source of GSH in the cytosol and mitochondria apart from the primary biosynthesis source. Hence, our observed PQ-evoked GSH depletion could be a result

of (1) decreased/inhibition of GSH synthesis, (2) increased GSH utilization, or (3) enhanced PQ-induced GR inhibition. Pretreatment with chrysin's partial mitigation of the decline in GR activity suggests its potential role in supporting GSH regeneration and subsequent increased chemical and metal detoxification (Franco *et al*., 2008).

Free radicals oxidize polyunsaturated fatty acids in cell membranes, including testis, releasing highly reactive compounds such as malondialdehyde (MDA), a byproduct of lipid peroxidation. Increased MDA suggests oxidative stress and can elicit cellular membrane damage, altering membrane permeability and ion balance and causing neurodegeneration and cell death (Peña-Bautista *et al*., 2019). The significant reduction of MDA levels by chrysin demonstrates its ability to lessen oxidative stress.

CONCLUSION

By scavenging free radicals, restoring antioxidant defense mechanisms and attenuating oxidative damage, chrysin supplementation offers a potential therapeutic strategy for mitigating paraquat-induced reproductive toxicity, which could be further explored in the translational potential of chrysin-based interventions in clinical settings.

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