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Original Article

Fresh palm oil improves impaired renal function in phenylhydrazine-induced anaemic Wistar rats via its anti-anaemic effect and modulation of expressions of pro-oxidant/antioxidants, inflammatory cytokines and caspase-3 in the kidneys

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ABSTRACT

Introduction: Phenylhydrazine (PHZ)-induced anaemia is associated with oxidative damage to erythrocytes and impaired renal function. Fresh palm oil (FPO) is a rich source of antioxidants and preserves renal function. This study investigated the possible reno-protective effects of FPO in PHZ-induced anaemic Wistar rats.

Methods: Eighteen male Wistar rats were randomly assigned into normal control (NC), anaemic control (AC; 60mg/kg of PHZ) and anaemic treated (A+FPO; 60mg/kg of PHZ + 15% FPO diet) groups. PHZ was administered twice consecutively at 48h interval while FPO diet was given throughout the study period (14 days).

Results: Erythrocyte count, haemoglobin concentration and haematocrit were increased in A+FPO compared with AC. FPO improved imbalances in the levels of glucose and electrolytes (Na^+, Cl^-, K^+, HCO_3^-) in serum and urine of the anaemic rats. Urea and creatinine concentrations were decreased in serum and increased in urine in the A+FPO relative to AC. Proteinuria was decreased in A+FPO compared with AC. The levels of malondialdehyde, pro-inflammatory cytokines and caspase-3 were down-regulated in A+FPO compared with AC while interleukin-10 concentration, enzymatic antioxidants activities and total antioxidant capacity were up-regulated in A+FPO compared with AC.

Conclusion: FPO demonstrated anti-anaemic effect and improved the impaired renal function associated with PHZ-induced anaemia.

Keywords:
Anaemia
Fresh palm oil
Haemolysis
Phenylhydrazine
Renal function

Introduction

Anaemia is a clinical condition characterized by a marked decrease in haemoglobin concentration resulting in decreased oxygen-carrying capacity of blood to body cells and tissues. The incidence of anaemia is higher in developing and underdeveloped countries than in devel-

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oped countries due to several factors including nutrient deficiency, recurrent infections and high prevalence of blood parasites like plasmodium and trypanosomes (Korubo-Owiye et al., 1998; Sanni et al., 2005). Anaemia is a threat to the global population. It has been reported to affect 1.62 billion people worldwide (Stevens et al., 2015). Anaemia is of different types depending on the causative factor. For the purpose of this study, we shall concentrate on only haemolytic anaemia. Haemolytic anaemia is the type of anaemia caused by haemolysis of the erythrocytes. It reduces oxygen transfer capacity and increases blood levels of iron which negatively affects other physiological processes in the body (Biswas et al., 2005). The haemolytic process is associated with oxidative stress on erythrocyte (Sochaski et al., 2002). Haemolysis can result from chemical interaction with sulfhydryl group, immune mechanisms, inhibition of certain enzymes, fragmentation of erythrocytes as they traverse the platelet-fibrin mesh or from poorly defined mechanisms (Stevens et al., 2015). Many researchers, in an attempt to study haemolytic anaemia and its associated effects, use the chemical, phenylhydrazine (PHZ), to induce anaemia.

PHZ is one of the most potent carcinogens belonging to the hydrazine family of molecules (Parodi et al., 1981). It was the first hydrazine derivatives that Hermann Emil Fischer characterised in 1875 (Berger, 2007). Its derivatives were initially used as antipyretics but because of their destructive effect on erythrocytes, their use was discontinued. PHZ intoxication causes oxidative damage to erythrocytes (Clemens et al., 1984) resulting in haemolytic anaemia with the involvement of spleen and liver (Stern, 1989; WHO, 2000). It damages the cell membrane, producing gradual haematological alterations, inflammatory mediators and increased red cells apoptosis (Vilsen and Nielsen, 1984; Fibach and Rachmilewitz, 2008). It also leads to hepatic and splenic iron overload (Ferrali et al., 1997). PHZ has been reported to cause lipid peroxidation in liver, kidney and spleen of mice (Ozcan et al., 2007). It induces oxidative damage to haemoglobin (Augusto et al., 1982), membrane phospholipids and proteins in erythrocytes of humans (Arduini et al., 1989; Hashmi and Saleemuddin, 1996). PHZ-induced haemolytic anaemia appears to result from oxidative damage to erythrocyte proteins rather than to interactions of membrane lipids (McMillan et al., 2005). PHZ's interaction with haemoglobin forms oxidised derivatives and free radicals of hydrazine which cause generation of hydrogen peroxide and destruction of the haemoglobin pigment (Ozcan et al., 2007). The PHZ-induced haemolytic anaemia occurs within 48h after lysis of erythrocytes (Berger, 2007). Aside decreased erythrocyte and haemoglobin concentrations, PHZ intoxication has been reported to decrease leucocyte, lymphocyte and thrombocyte counts, increase serum urea concentration and cause histopathological alterations of the kidneys of Wistar rats (Kale et al., 2019). Also, oxidative stress-induced haemodynamic disturbance and vascular dysfunction (Luangaram et al., 2007) as well as derangement in electrolytes (Berger, 2007; Beshel et al., 2019) have been reported to occur following PHZ intoxication. Decreased glomerular filtration rate (Haymann et al., 2010), albuminuria and proteinuria (Day et al., 2012; Novelli et al., 2014) have also been reported to occur following haemolysis.

Palm oil, which originated from Africa and now spreading throughout most of the world (Mukherjee and Mitra, 2009), is one of the most widely used cooking oils in Central and West Africa (Ani et al., 2015). It is extracted from the mesocarp of oil palm (*Elaeis guineensis*) fruit. In Nigeria and most parts of Africa, palm oil is consumed mainly in its fresh and thermally oxidised forms (Ebong et al., 1999). Palm oil, in its fresh form, is obtained from the pulp of the palm fruits by squeezing and boiling at low temperature to remove debris - when it has neither been refined nor undergone thermo- and/or photo-oxidation. Palm oil contains antioxidants and other phytonutrients with nutritional and health benefits including carotenoids, tocopherols, tocotrienols, phytosterol, phenolic compounds (Sambanthamurthi et al., 2000; Edem, 2002), palmitic, stearic, monounsaturated oleic and polyunsaturated linoleic acids (Mortensen, 2005; Kok et al., 2011; Sampaio et al., 2011) as well as squalene and coenzyme Q10 (Loganathan et al., 2017). The characteristic red color of fresh palm oil (FPO) is due to its abundance of alpha- and beta-carotenes which can make up 0.08% (w/w) of the crude oil (Orozco et al., 2006). The natural antioxidants in palm oil make it a very vital substance for human consumption as they play protective roles in atherosclerosis, Alzheimer's disease, arthritis, cellular ageing (Sutapa and Analava, 2009), hypertension, hypercholesterolaemia, arteriosclerosis and skin and breast cancers (Imoisi et al., 2015). FPO has been reported to reduce the prevalence

of maternal anaemia (Radhika et al., 2003) and not have any significant effect on erythrocyte count, packed cell volume, haemoglobin concentration (Mesembe et al., 2004), plasma and urine electrolytes (Na^+, K^+, Cl^- and HCO_3^-) (Beshel et al., 2018) as well as blood pressure, glomerular filtration rate and renal plasma flow (Beshel et al., 2013).

From the foregoing, it is clear that PHZ causes oxidative damage to erythrocytes resulting in haemolytic anaemia. It is also clear that PHZ intoxication causes lipid peroxidation in the kidneys and causes derangement in electrolytes implying an impairment in renal function. Haemolysis itself is associated with decreased glomerular filtration rate, albuminuria and proteinuria. On the other hand, FPO is a rich source of antioxidants and has been reported to preserve renal function and not have any significant effect on erythrocyte count and haemoglobin concentration. Whether FPO can also protect the erythrocyte from PHZ-induced oxidative damage remains unknown. If at all FPO can, can it also preserve renal function in PHZ-induced haemolytic anaemic state? This study was therefore undertaken to provide answers to these questions.

Material and methods

Purchase of fresh palm oil and formulation of fresh palm oil diet

Ten litres of fresh palm oil were purchased directly from the palm oil mill at Odukpani Local Government Area of Cross River State, Nigeria and immediately stored inside a black container. The container was kept in a cool dry room and not exposed to sunlight or heat. The FPO diet was formulated as previously described (Beshel et al., 2018). Briefly, 15g of the FPO was mixed with 85g of rat chow to make 15% FPO diet.

Laboratory animals

Approval to conduct the study was granted by the Animal Research Ethics Committee of Faculty of Basic Medical sciences, University of Calabar, Nigeria. Eighteen male Wistar rats (165–180g) aged 8–9 weeks old were used for the study. The animals were bought from Department of Agriculture, University of Calabar and kept in individual metabolic cages in the animal room of our Department. The 1985 guidelines of the National Institute of Health publication for laboratory animal were followed in the handling of the rats. This was confirmed by the above Animal Research Ethics Committee. The rats were subjected to 12h light/dark cycle and acclimatised for two weeks before being induced with anaemia and allowed to feed on FPO diet.

Experimental design

The eighteen rats were randomly assigned into three groups with six rats per group: 1- normal control group (NC) that received rat chow and tap water; 2- anaemic control group (AC) that was induced with anaemia and left untreated for 14 days; 3- anaemic treated group (A+FPO) that was induced with anaemia and fed 15% FPO diet for 14 days.

Rats in all groups were administered a physiological solution (0.2ml normal saline, IP) for 14 days. Anaemia was induced by intraperitoneal injection of 60mg/kg of phenylhydrazine for two consecutive times at 48h interval. Twenty-four hours after the second dose, haemoglobin (Hb) concentration was measured and rats whose Hb concentration was <11.5 g/dl and whose erythrocyte count and haemoglobin concentration reduced by at least 30% were considered anaemic (Fernández et al., 2010; Jaiswas et al., 2014). Prior to induction of anaemia, Hb concentration was measured in all rats using Sahli's apparatus. Hb concentration was also measured immediately after induction of anaemia using the same apparatus and at the end of the study using automated cell counter (Coulter Electronics, Bedfordshire, UK).

Harvesting of kidneys and collection of blood and urine samples

At the end of the study period, the rats were anaesthetised with 60mg/kg pentobarbital and sacrificed. Blood samples of the animals were collected via cardiac puncture using 5ml syringes attached to 21G needles into ethylenediaminetetracetate (EDTA) vials and gently agitated to ensure uniform spread of EDTA. Some portions of the blood samples were also introduced into plain sample bottles. The samples in the EDTA vials were used for determination of erythrocyte count, Hb concentration and haematocrit (packed cell volume, PCV) while the ones in the plain sample bottles were allowed for 1h to clot and then centrifuged (B-Bran Scientific and Instrument Company, England) at 1000g for 15min and used for determination of serum biomarkers of renal function.

To collect urine, a small lower abdominal incision was

made. Through this incision, a short self-retaining catheter (pp100, polythene tubing) was used to cannulate the urinary bladder. This was followed by ligation of the urethra to prevent voiding of urine. After a sixty-minute equilibration period, urine samples were collected into pre-weighed sample bottles for another 60min and stored until when needed. Both kidneys of the rats were also harvested, cleared of connective tissues, weighed, homogenised and used for determination of oxidative stress biomarkers.

Measurement of body and kidney weights

Body weight was measured at the commencement and end of the feeding period using a sensitive electronic weighing balance (Scout Pro, Ohaus Corporation). The combined absolute weight of both kidneys of each rat was also measured using this same weighing balance. The relative weight (RW) of both kidneys of each rat was calculated using the relationship below:

RW of both kidneys= (combined absolute weight of both kidney/final body weight) × 100%

Determination of erythrocyte count, Hb concentration and PCV

Erythrocyte count, Hb concentration (at the end of feeding period) and PCV were determined using automated cell counter (Coulter Electronics, Bedfordshire, UK) having standard calibrations in line with the manufacturer's instructions.

Serum and urine assays

The concentrations of Na^+, K^+ and Cl^- in serum and urine were determined using ion-selective electrolyte analyzer (Biolyte 2000/ BioCare Corporation, Hsinchu 300, Taiwan). To measure HCO_3^- concentration, we first of all measured the pH and partial pressure of carbon dioxide (pCO_2) in the samples using a gas analyser (Rapidlab-1265, Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The HCO_3^- concentration was then computed from the measured pH and pCO_2 using the Henderson – Hasselbalch equation shown below (Ramsay, 1965): $pH = 6.1 + \log([HCO_3^-]/pCO_2 \times 0.03)$.

Serum and urine concentrations of urea were determined using Berthelot's reaction as described by Kaplan and Teng (1982) while serum and urine concentrations of creatinine were determined as described by Estridge et al. (2000) using the Reflotron Dry Chemistry Analyzer. Glucose levels in serum and urine samples were measured in triplicate with a Beckman Glucose Analyzer II (Beckman, Fullerton, Calif., USA). Serum and urine levels of albumin, globulin and total protein were determined using commercially available kits and an automatic biochemistry analyzer (Mindray BS-800, Shenzhen, China).

Preparation of kidney homogenate

Each rat's kidney was homogenised in Tris-HCl buffer (pH 7.4) using a Potter Elvehjem homogenizer. The homogenate (10%) was centrifuged at 1000g for 20min at 4°C. The aliquots were immediately frozen and stored at -80°C until they were used for analysis of oxidative stress biomarkers, pro- and anti-inflammatory cytokines and caspase-3 protein level.

Assessment of lipid peroxidation and antioxidants activities in the kidneys

The method of Ohkawa et al. (1979) was used to quantify lipid peroxidation as malondialdehyde (MDA) concentration. Here, 150µl of the sample was mixed with 300µl of 10% trichloroacetic acid. This was followed by centrifugation (4°C) of the mixture for 10min at 1000g. Thereafter, 300µl of the supernatant obtained was introduced into a test tube that contained 300µl of 67% thiobarbituric acid. The resulting content was incubated at 100°C for 25min. Five minutes after the solution got cooled, a pink color appeared due to MDA-TBA reaction and was evaluated using a spectrophotometer at 535nm. Catalase (CAT) activity was assessed using the method described by Clairborne (1995). In this assay, H_2O_2 was used as substrate. Xylenol orange dye was used to measure the oxidation of Fe^{+2} to Fe^{+3} mediated by H_2O_2 using a spectrophotometer at 560nm. Superoxide dismutase (SOD) activity and glutathione (GSH) level were determined using the methods of Misra and Fridovich (1972) and Annuk et al. (2001) respectively. For total antioxidant capacity (TAC), the method of Koracevic et al. (2001) was employed. This assay is based on Fenton Reaction (reduction of the formation of thiobarbituric acid reactive substances following the reaction of Fe-EDTA complex and H_2O_2). Uric acid was used as the standard for this assay (Erejuwa et al., 2010) and the results were expressed as nmol/mg protein.

Determination of levels of pro- and anti-inflammatory

TABLE 1: Body and kidney weights in the different Experimental groups

Group	IBW (g)	FBW (g)	BWC (g)	AW (Both kidneys) (g)	RW (Both kidneys) (%)
NC	187.50 ± 2.62	210.33 ± 3.01	22.83 ± 2.59	0.59 ± 0.03	0.28 ± 0.02
AC	190.67 ± 2.81	177.67 ± 2.26^{+}	-13.00 ± 4.13^{+}	0.80 ± 0.03^{+}	0.45 ± 0.02^{+}
A+FPO	189.83 ± 3.16	195.33 ± 3.32$^{+,\#}$	5.50 ± 3.89$^{+,\#}$	0.68 ± 0.04$^{\#}$	0.35 ± 0.03$^{+,\#}$

Values are mean±SEM, n = 6. ^{+}P< 0.05 vs NC, $^{\#}P$< 0.05 vs AC. IBW: initial body weight, FBW: final body weight, BWC: body weight change, AW: absolute weight, RW: relative weight.

cytokines in the kidneys

The levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β and IL-10 in the kidney homogenates were determined using ELISA kits (BioAssay Systems, Hayward, CA, USA) following the protocol set by the manufacturer.

Determination of caspase-3 activity in the kidneys

We employed the modified Fluorometric CaspACE Assay System (Promega) in the detection of caspase-3 activity in the kidneys of the rats. A volume of the supernatant (equivalent to 100mg) obtained after homogenisation was assayed for the activity of caspase-3 by the ability to cleave the fluorogenic substrate Ac-DEVDAMC. The caspase-3 inhibitor Ac-DEVD-CHO was used to determine the specificity of the assay by adding to the sample 30min before the substrate. By using a fluorescence microplate reader (SOFTmax PRO, Molecular Devices Corp., Sunnyvale, CA) which uses an excitation wavelength of 360nm and an emission wavelength of 460nm, we monitored the proteolytic cleavage of the substrates. The fluorescence intensity was calibrated with standard concentrations of AMC and the activity of caspase-3 was calculated from the slope of the recorder trace and expressed in ρmol/min/µg protein (Yang et al., 2001).

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics software, version 20. The results are presented as mean±SEM. Normality of the data was tested using Shapiro-Wilk test while homogeneity of variance was tested using Levene's test. After confirming normality of the distribution and homogeneity of variance, One-way analysis of variance (ANOVA) was therefore employed to analyse the data and post hoc test (Least Square Difference) was performed to make comparisons. Values of P<0.05 were considered significant.

Results

Effects of FPO diet on body and kidney weights in PHZ-induced anaemic Wistar rats

The initial body weight was not significantly different among the groups. At the end of the study period, the final body weight decreased (P<0.05) in the anaemic control rats and the anaemic rats treated with FPO compared with the normal control rats. However, the final body weight increased significantly in the anaemic rats treated with FPO compared with the anaemic control rats. The change in body weight of the rats in all groups followed a similar pattern as their final body weight (Table 1).

The combined absolute and relative kidney weights of the anaemic control rats increased significantly compared with the normal control rats. In the anaemic rats fed FPO diet, these combined absolute and relative kidney weights were decreased (P<0.05) in comparison with the anaemic control rats (Table 1).

Effects of FPO diet on erythrocyte count, Hb concentration and PCV in PHZ-induced anaemic Wistar rats

Rats in the anaemic control group had significantly decreased erythrocyte count and PCV compared with the normal control rats. Interestingly, erythrocyte count and PCV in the anaemic rats that received FPO diet increased (P<0.05) in comparison with the anaemic control rats. Hb concentration was measured before and after induction of anaemia and at the end of the study. There was no significant difference in Hb concentration before induction. After induction, rats with at least 30% decrease in Hb concentration were considered anaemic. As expected after PHZ injection, Hb concentration in rats in the anaemic control group decreased (P<0.05) and by 30.4% compared with the normal control rats. Hb concentration in the anaemic rats fed FPO diet, which was increased (P<0.05) compared with the anaemic control rats, was comparable with the normal control rats with just a difference of about 2.3%. This trend

TABLE 2: Erythrocyte count, Hb concentration, and PCV in the different experimental groups.

Group	RBC (million/µl)	Hb (g/dl)			PCV (%)
		Before Induction	After Induction	After Treatment	
NC	7.48 ± 0.76	15.10 ± 0.36	14.72 ± 0.49	14.45 ± 0.25	41.38 ± 1.53
AC	3.88 ± 0.41^{+}	14.92 ± 0.51	10.27 ± 0.42^{+}	9.82 ± 0.45^{+}	24.80 ± 1.35^{+}
A+FPO	6.02 ± 0.68$^{\#}$	14.60 ± 0.47	14.37 ± 0.40$^{\#}$	14.28 ± 0.31$^{\#}$	39.12 ± 2.29$^{\#}$

Values are mean±SEM, n=6. $^{+}P<0.05$ vs NC, $^{\#}P<0.05$ vs AC.

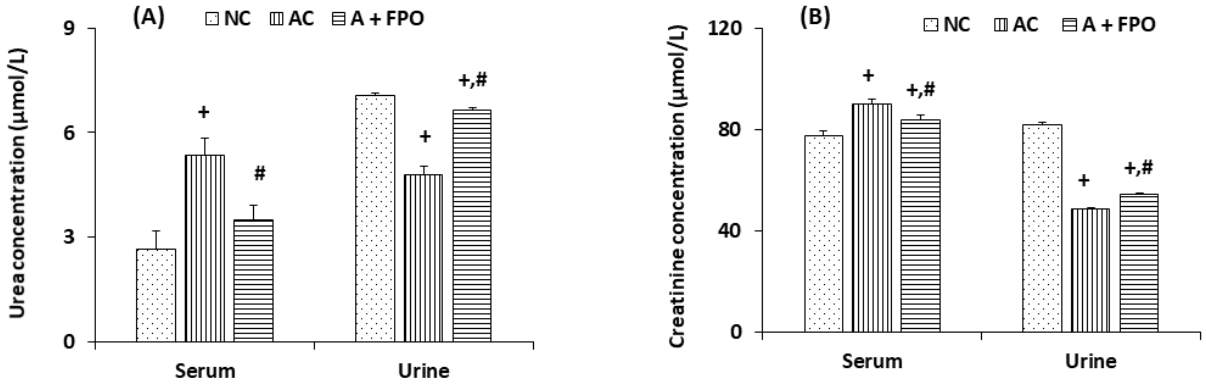

FIGURE 1. Serum and urine concentrations of (A) urea and (B) creatinine in the different experimental groups. Values are mean±SEM, n=6. $^{+}P<0.05$ vs NC, $^{\#}P<0.05$ vs AC.

was maintained at the end of the study indicating the sustenance of the PHZ-induced anaemia. Here, Hb concentration in the anaemic control rats was still decreased ($P<0.05$) and by 32.04% compared with the normal control rats. Hb concentration in the anaemic rats fed FPO diet was increased ($P<0.05$) compared with the anaemic control rats but was just only 1.18% lower than the control rats (Table 2).

Effects of FPO diet on serum and urine concentrations of electrolytes in PHZ-induced anaemic Wistar rats

Table 3 shows the concentrations of electrolytes (Na^+, Cl^-, K^+ and HCO_3^-) in serum and urine in the different experimental groups. Serum Na^+ concentration decreased significantly in AC and A+FPO compared with NC. However, there was an increase ($P<0.05$) in serum Na^+ concentration in the A+FPO in comparison with AC. Meanwhile, urine concentration of Na^+ increased ($P<0.05$) in AC and A+FPO compared with NC but was significantly decreased in A+FPO compared with AC. Serum and urine concentrations of Cl^- followed the same trend as Na^+ except that the decrease in serum Cl^- concentration in A+FPO in comparison with NC was not significant.

On the other hand, serum K^+ concentration increased significantly in the anaemic control rats and anaemic rats fed FPO diet in comparison with the normal control rats. However, it decreased ($P<0.05$) in A+FPO compared with AC. Urine K^+ concentration was significantly decreased in AC in comparison with NC. Serum HCO_3^- concentration was not significantly different among the groups but urine HCO_3^- concentration decreased significantly in the anaemic control rats in comparison with the normal control rats.

Effects of FPO diet on serum and urine concentrations of urea and creatinine in PHZ-induced anaemic Wistar rats

Serum urea concentration was significantly increased in the anaemic control group in comparison with the normal control group. However, there was a significantly decreased urea concentration in the anaemic rats fed FPO diet compared with the anaemic control rats. Urea concentration in urine was however decreased ($P<0.05$) in AC and A+FPO compared with NC. Urine urea concentration was increased significantly in A+FPO in comparison with AC (Figure 1).

Serum creatinine concentration was significantly increased in the anaemic control rats and anaemic rats fed FPO diet compared with the normal control rats. But it

TABLE 3: Serum and urine concentrations of electrolytes in the different experimental groups.

Group	Na^+ (mmol/l)		Cl^- (mmol/l)		K^+ (mmol/l)		HCO_3^- (mmol/l)	
	Serum	**Urine**	**Serum**	**Urine**	**Serum**	**Urine**	**Serum**	**Urine**
NC	142.17 ± 1.35	26.67 ± 0.49	108.17 ± 1.08	21.50 ± 0.99	3.68 ± 0.21	34.00 ± 1.26	24.00 ± 0.58	12.17 ± 0.87
AC	133.33 ± 0.76$^+$	33.17 ± 0.60$^+$	99.83 ± 1.42$^+$	29.33 ± 1.09$^+$	7.18 ± 0.27$^+$	28.67 ± 1.12$^+$	22.33 ± 0.95	9.83 ± 0.48$^+$
A + FPO	138.50 ± 1.20$^{+,\#}$	29.17 ± 0.75$^{+\#}$	105.67 ± 1.41$^\#$	25.67 ± 0.76$^{+\#}$	5.25 ± 0.24$^{+\#}$	30.83 ± 0.91	22.67 ± 0.49	10.33 ± 0.67

Values are mean±SEM, n=6. $^+P<0.05$ vs NC, $^\#P<0.05$ vs AC.

TABLE 4: Serum and urine concentrations of glucose in the different experimental groups.

Group	Glucose (mmol/l)	
	Serum	**Urine**
NC	3.767 ± 0.250	0.21 ± 0.005
AC	6.367 ± 0.440$^+$	0.26 ± 0.007$^+$
A+FPO	5.100 ± 0.312$^{+,\#}$	0.21 ± 0.007$^\#$

Values are mean±SEM, n=6. $^+P<0.05$ vs NC, $^\#P<0.05$ vs AC.

however decreased significantly in the anaemic rats fed FPO diet in comparison with the anaemic control rats. Creatinine concentration in urine decreased significantly in AC and A+FPO in comparison with NC. It however increased significantly in A+FPO compared with AC (Figure 1).

Effects of FPO diet on serum and urine glucose concentrations in PHZ-induced anaemic Wistar rats

Table 4 shows serum and urine glucose concentration in the different experimental groups. Serum glucose concentration was increased ($P<0.05$) in the anaemic control and anaemic treated groups compared with the normal control group. But it was decreased significantly in the anaemic treated group in comparison with the anaemic control group. While urine glucose concentration was significantly increased in the anaemic control group in comparison with the normal control group, it however decreased ($P<0.05$) in the anaemic treated group compared with the anaemic control group.

Effects of FPO diet on serum and urine protein concentrations in PHZ-induced anaemic Wistar rats

Total protein and albumin concentrations decreased significantly in the anaemic control and anaemic treated groups compared with the normal control group. They were however increased ($P<0.05$) in the anaemic group fed FPO diet in comparison with the anaemic control group. Serum globulin concentration followed the same trend except that its decrease in the anaemic treated group in comparison with the normal control group was not significant (Figure 2). In the urine, total protein, albumin and globulin concentrations were all increased ($P<0.05$) in the anaemic control and anaemic treated groups in comparison with the normal control group. All three decreased ($P<0.05$) in the anaemic treated group in comparison with the anaemic control group (Figure 2).

Effects of FPO diet on kidney oxidative stress biomarkers in PHZ-induced anaemic Wistar rats

Figure 3 shows the results for oxidative stress biomarkers in the kidneys of rats in the different experimental groups. MDA level was up-regulated ($P<0.05$) in the anaemic control group compared with normal control group but was down-regulated ($P<0.05$) in the anaemic treated group in comparison with the anaemic control group. SOD activity was down-regulated ($P<0.05$) in AC compared with NC but was up-regulated ($P<0.05$) in A+FPO in comparison with AC. CAT activity and TAC were significantly decreased in the anaemic control and anaemic treated groups compared with the normal control group. Interestingly, both were increased ($P<0.05$) in the anaemic treated group in comparison with the anaemic control group. Glutathione level was not significantly different among the groups.

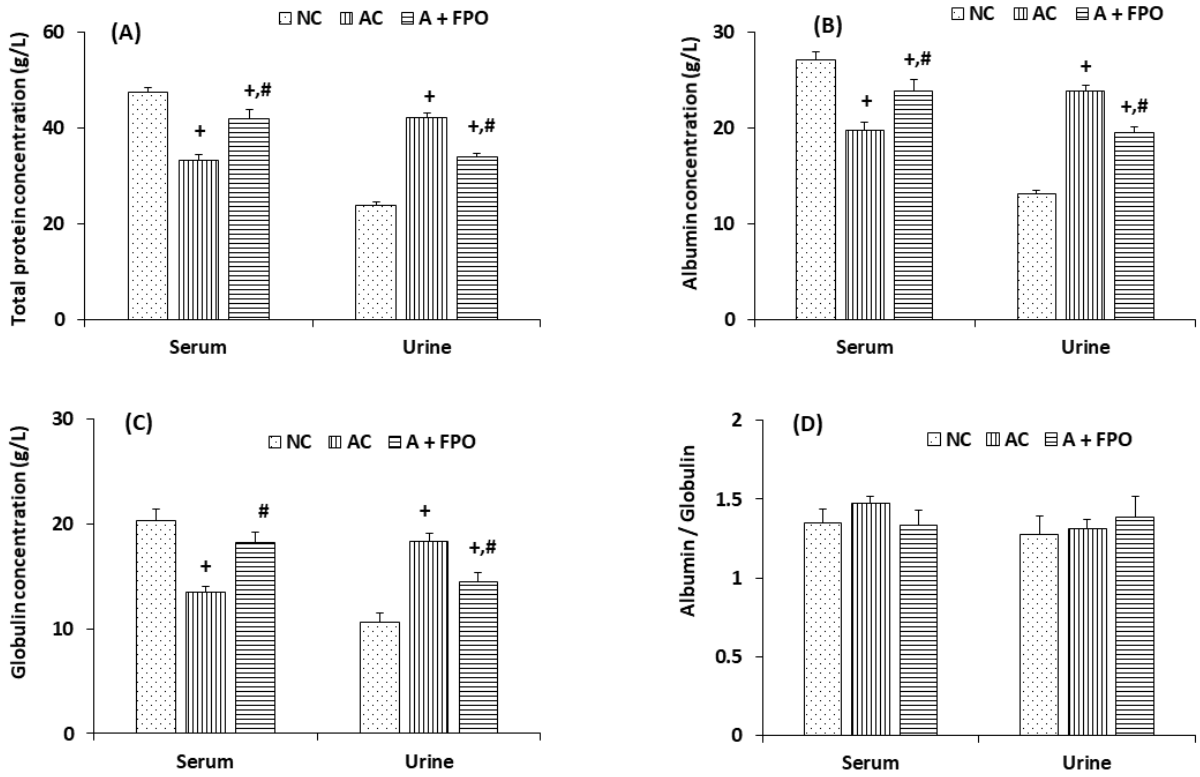

FIGURE 2. Serum and urine concentrations of proteins in the different experimental groups. (A) Total protein, (B) albumin, (C) globulin and (D) albumin/globulin. Values are mean±SEM, n=6. $^{+}P<0.05$ vs NC, $^{\#}P<0.05$ vs AC.

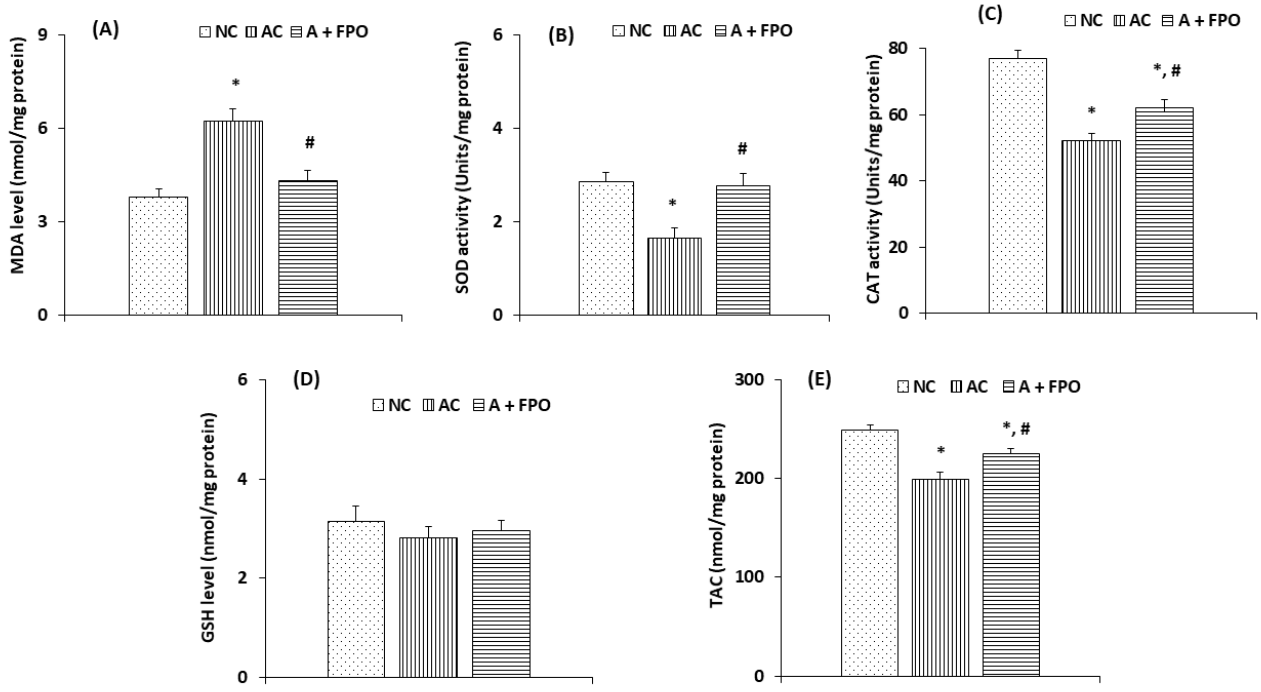

FIGURE 3. (A) MDA level, (B) SOD activity, (C) CAT activity, (D) GSH level, and (E) TAC in the different experimental groups. Values are mean±SEM, n=6. +P<0.05 vs NC, #P<0.05 vs AC.

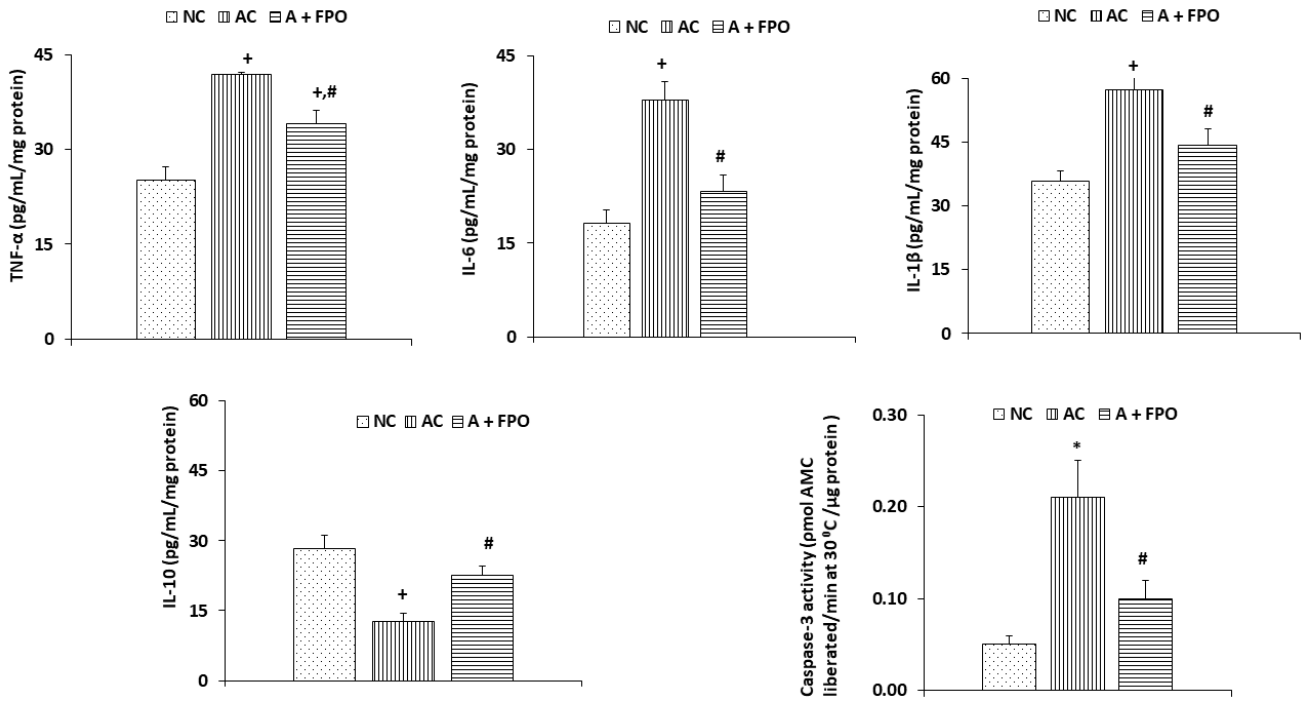

FIGURE 4. Pro- and anti-inflammatory cytokines and caspase-3 activity in the different experimental groups. Values are mean±SEM, n=6. +P<0.05 vs NC, #P<0.05 vs AC.

Effects of FPO diet on pro- and anti-inflammatory cytokines and caspase-3 activity in kidneys of PHZ-induced anaemic Wistar rats

Figure 4 shows the levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), anti-inflammatory cytokine (IL-10) and caspase-3 activity in the different experimental groups. All pro-inflammatory cytokines were up-regulated (P<0.05) in the anaemic control group compared with the normal control group but were downregulated (P< 0.05) in the anaemic treated group in comparison with the anaemic control group. On the other hand, IL-10 was down-regulated (P<0.05) in the anaemic control group compared with the normal control group and up-regulated (P<0.05) in the anaemic treated group in comparison with the anaemic control group. Caspase-3 activity was up-regulated (P<0.05) in the anaemic control group in comparison with the normal control group. It was however down-regulated (P<0.05) in the anaemic treated group compared with the anaemic control group.

Discussion

The regulation of acid-base balance and concentrations of electrolytes and other solutes like glucose and urea are basic roles of the kidneys to maintain homeostasis. These functions of the kidneys can be compromised by several factors including kidney disease and haemolytic anaemia. In a previous study, derangement in concentrations of electrolytes was reported following PHZ-induced anaemia (Beshel et al., 2019). Other investigators have documented that PHZ intoxication is associated with lipid peroxidation (Ozcan et al., 2007) and histopathology (Kale et al., 2019) of the kidneys suggesting that the haemolytic anaemia caused by PHZ, does not only affect the erythrocytes but also affects the kidneys directly. Therefore, the use of antioxidants or antioxidants-containing substances may offer some degree of protection against impaired renal function occasioned by PHZ intoxication. In the present study, we investigated the possible reno-protective effect of fresh palm oil (having high antioxidants content) on renal function in PHZ-induced anaemic Wistar rats and its underlying mechanisms of action.

PHZ-induced anaemia resulted in decreased body weight and increased kidney weight, in line with Kale et al. (2019) who reported that PHZ-induced anaemia resulted in increased kidney weight. However, following consumption of FPO diet, body weight was improved and kidney weight tended toward normal in the anaemic rats (Table 1).

The decreased Hb concentration observed in this

study following PHZ intoxication confirms the successful induction of the haemolytic anaemia. Moreover, erythrocyte count and PCV also decreased consistent with previous studies (Kolawole et al., 2017; Osafanme et al., 2019). PHZ itself is known to cause oxidative damage to erythrocytes. Luangaram et al. (2007) have shown PHZ intoxication to result in increased plasma concentrations of MDA and nitric oxide metabolites, superoxide radical production in blood cells and decreased whole blood GSH level in rats. As erythrocytes are the determinants of PCV which represents the percentage of erythrocytes in blood it is possible that apart from erythrocytes destruction, PHZ intoxication affected the bone marrow resulting in decreased erythropoiesis. It is also possible that PHZ intoxication affected erythropoietin production by the kidneys which impaired erythropoietin's ability to stimulate the bone marrow for erythropoiesis to take place (Kale et al., 2019). PHZ has been reported to affect the erythropoietin receptor responsible for erythrocyte maturation (Li et al., 2003). However, following FPO diet consumption, rythrocyte count, Hb concentration and PCV were significantly improved and tended toward normal levels suggesting that FPO was able to protect against PHZ-induced anaemia (Table 2). This demonstrates the anti-anaemic effect of FPO, which could be due to its rich antioxidant content.

Consistent with previous studies (Berger, 2007; Cuhadar, 2011; Palmer and Clegg, 2016; Beshel et al., 2019), the present study shows that haemolytic anaemia causes derangement in the levels of electrolytes. In the anaemic control rats were observed moderate hyponatraemia, hypochloridaemia and hyperkalaemia but HCO_3^- was not negatively affected. The hyponatraemia could be said to be hypovolaemic as a result of deficiency in mineralocorticoids, with urine Na^+ concentration greater than 20mmol/l as was the case in the present study (Sahay and Sahay, 2014). Chloride concentrations followed a similar pattern as Na^+ in order to balance the electrochemical equivalent as both are the major extracellular fluid electrolytes. In normal condition, when there is hyponatraemia and hypochloridaemia, there is a corresponding decrease in urinary excretion of Na^+ and Cl^-. Similarly, in hyperkalaemia, urinary excretion of K^+ is increased. But this is not the case in the present study as there was increased urinary excretion of Na^+ and Cl^- and decreased urinary excretion of K^+ in the anaemic control rats in comparison with the normal control rats. This is typical of haemolytic anaemia where there is usually hyperkalaemia but low K^+ in urine (Berger, 2007). This suggests that the PHZ-induced anaemia impaired renal function which resulted in the derangement of electrolytes. The decreased urinary excretion of K^+ observed in this study despite the hyperkalaemic state might be as a result of decreased distal delivery of Na^+, mineralocorticoid deficiency and/or abnormal function of the cortical collecting tubule (Beshel et al., 2018). The decreased serum and urine concentrations of HCO_3^- observed in the anaemic control rats is due to the hyponatraemia as alkalosis has been reported to occur with hyponatraemia (DeCaux et al., 2003). However, following consumption of FPO diet by anaemic rats, the concentrations of electrolytes in both serum and urine were improved (Table 3).

Urea and creatinine are also markers for assessing renal function. Urea is the by-product of erythrocyte metabolism while creatinine is one of the waste products in the blood undergoing continuous filtration by the kidneys and excretion into the urine. Normally, their concentrations are lower in blood and higher in urine. Higher than normal concentrations of urea and creatinine in the blood as it is the case with the anaemic control rats in this study, are indicative of renal insufficiency and disease (Luber, 1988). The increased urea concentration following PHZ intoxication is consistent with Kale et al. (2019) and also reflects the haemolytic destruction of erythrocytes observed in the anaemic control rats as blood urea concentration increases following erythrocyte destruction. Normally, when blood urea and creatinine concentrations are high, there should be corresponding increases in their urine concentrations. In the anaemic control rats, the urea and creatinine concentrations in the urine were lower than their values in serum and were significantly decreased compared with the normal control rats (Figure 1) suggesting an impairment in renal function. But with consumption of FPO diet, the urea and creatinine concentrations in serum and urine improved in the anaemic rats and tended toward normal values indicating that the FPO diet offered reno-protective effect against PHZ intoxication. Consumption of FPO diet has been previously reported to maintain normal kidney architecture and not have any significant effect on serum urea and creatinine concentrations in rabbits (Ani et al., 2015) as well as blood pressure, glomerular filtration rate and renal plasma flow in rats

(Beshel et al., 2013).

To further assess renal function, we also measured the serum and urine levels of glucose and proteins as the kidneys are responsible for regulation of the concentrations of these solutes. We observed that although glucose concentration in urine was very low, its level was significantly increased in the anaemic control rats compared with the normal control rats. The increased serum glucose level observed in the anaemic control rats suggests that the PHZ-induced anaemia interfered with glucose metabolism thus causing hyperglycaemia (Table 4). The increased proteinuria observed in the anaemic control rats also confirms the impairment in renal function occasioned by PHZ intoxication. Albumin, globulin and total protein concentrations decreased in the serum of the anaemic rats in comparison with the normal control rats whereas their concentrations in urine increased relative to normal control and the values in urine were higher than those in serum (Figure 2). These results are similar to the work of Kale et al. (2019) which documents slight decreases in serum albumin and total protein concentrations following PHZ intoxication. However, FPO diet fed to anaemic rats tended to restore the levels of these proteins toward normal thus also suggesting its reno-protective effect (Figure 2).

We can confirm from our study that the impaired renal function associated with PHZ-induced anaemia was mediated by oxidative stress, inflammation and apoptosis in the kidneys. The up-regulation of MDA level as well as down-regulation of TAC and activities of SOD and CAT in the anaemic control rats suggests that the PHZ-induced anaemia resulted in renal oxidative stress (Ozcan et al., 2007; Kolawole et al., 2017). The decreased SOD and CAT activities and TAC caused destruction of the lipid membranes by accumulation of H_2O_2 which caused the elevation of the MDA level. In the normal situation, generation of reactive oxygen species causes the deployment of SOD which catalyzes the dismutation of superoxide radical to H_2O_2. The H_2O_2 is then converted to H_2O and O_2 by CAT (Aprioku, 2013). Interestingly, MDA level was downregulated and TAC, SOD and CAT activities increased in the anaemic rats fed FPO diet in comparison with the anaemic control rats suggesting the ability of the FPO diet to reduce lipid peroxidation and improve the antioxidant defense system thus combating the renal oxidative stress caused by PHZ intoxication. We attribute this reno-protective effect of FPO diet to its antioxidant content. FPO is known to contain carotenoids, tocopherols, tocotrienols, phenolic compounds and coenzyme Q10 (Sambanthamurthi et al., 2000; Edem, 2002; Loganathan et al., 2017).

In addition to oxidative stress, PHZ intoxication also resulted in inflammation in the kidneys. This is confirmed by the up-regulation of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and down-regulation of the anti-inflammatory cytokine, IL-10 in the kidneys of rats in the anaemic control group in comparison with rats in the normal control group. FPO diet however demonstrated anti-inflammatory effect in the kidneys of the anaemic rats as TNF-α, IL-6 and IL-1β were down-regulated and IL-10 up-regulated in the A+FPO in comparison with AC (Figure 4). This anti-inflammatory effect of FPO may also be due to its antioxidant content.

Caspase-3 is one of the down-stream effectors of the apoptotic process, the other being caspase-7. It is the most important effector enzyme in apoptosis and has been linked to the pathogenesis of different models of renal injury associated with apoptosis (Yang et al., 2001). Its activation is stimulated by stress stimuli such as reactive oxygen species and DNA damage within the cell through a cascade of reactions involving the release of cytochrome c from the mitochondria by B-cell lymphoma-2 associated X (Bax) and binding of the cytochrome c to other caspase-3-activating proteins (Udefa et al., 2020). Once activated, caspase-3 causes cell death. We observed in this study that, following PHZ intoxication, caspase-3 protein expression was up-regulated in the kidneys of the anaemic control rats in comparison with the normal control rats. This suggests that, PHZ-induced anaemia is associated with apoptosis in the kidneys which is partly responsible for the impaired renal function observed in the anaemic control group (Kale et al., 2019). However, consumption of the FPO diet by the anaemic rats resulted in decreased level of caspase-3 in the kidneys indicating the ability of FPO to combat the apoptotic process (Figure 4). This anti-apoptotic effect of FPO could also be attributed to its rich antioxidant content.

From the foregoing, it is clear that FPO diet exhibited anti-anaemic, anti-inflammatory and anti-apoptotic effects against PHZ-induced impairment in renal function. These properties of FPO diet can be attributed to the rich antioxidant content of FPO. The carotenoids, tocopherols and tocotrienols contained in FPO are known to

fight against varying degrees of oxidation in the body, improve cardiovascular health and protect against diseases such as atherosclerosis, arteriosclerosis, hypertension, cancer, ageing and Alzheimer's disease (Sutapa and Analava, 2009; Imoisi et al., 2015; Loganathan et al., 2017).

Conclusion

It is shown in this study that, PHZ-induced anaemia in Wistar rats is associated with oxidative stress, inflammation and apoptosis of the kidney tissues resulting in the impairment of renal function. FPO diet is capable of improving renal function in PHZ-induced anaemic rats via modulation of antioxidant activities and expression of inflammatory cytokines and caspase-3 in the kidneys.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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