IOSR Journal of Nursing and Health Science (IOSR-JNHS)
e-ISSN: 2320–1959.p- ISSN: 2320–1940 Volume 13, Issue 4 Ser.6 (July. – August. 2024), PP 19-25
www.iosrjournals.org

Renal Cytoarchitecture Is Restored And Proteinuria Reversed By Ferrous Sulphate In Rats Intoxicated With Phenylhydrazine

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Abstract
Background: *Phenylhydrazine is known to cause oxidative stress leading hemolytic anaemia because of the susceptibility of RBCs to oxidative stress. Other tissues, especially rapidly metabolising tissues get to be affected secondary to this. The damage to renal tissue usually causes proteinuria and glycosuria. Ferrous sulphate is one of the cheapest and most readily available hematinics. Since the damage to tissues is secondary to hemolytic anemia. Can the administration of ferrous sulphate reverse the damage to renal tissue and attendant proteinuria and glycosuria? The aim of this study is to find out the effect of ferrous sulphate on the renal cytoarchitecture and proteinuria and glycosuria in rats intoxicated with phenylhydrazine.*
Materials and Methods: *Sixteen (16) male Wistar rats weighing 200 – 250 grams were randomly divided into four groups namely: Group 1 – Normal control, Group 2 – Hematinic group (Fes): fed normal rat chow + tap water + ferrous sulphate (using an oral gavage at 75mg/kg bw); Group 3–Anaemic – treated group (AFes); administered Phenylhydrazine (PHZ) intraperitoneally for two consecutive days to induce anemia at a dose of 40mg/kg bw + normal rat chow + tap water + ferrous sulphate at 75mg/kg bw. Group 4 (Anu) – anemic control group: administered Phenylhydrazine (PHZ) intraperitoneally at a dose of 40mg/kg of bw + norma rat chow + tap water (as in group one). After 15 days, blood and urine samples were collected into sterile sample bottles for analysis. The kidneys were harvested and prepared for histological studies.*
Results: *There was a significant ($P<0.001$) increase in the urine glucose concentration when AFes, and Anu were compared with the control group. The urine Anu concentration was significantly ($P<0.001$) higher than Fes and AFes. The protein in the urine of Anu was significantly ($P<0.001$; $P<0.001$ and $P<0.01$) higher than that of control, Fes and AFes. The creatinine concentration in the plasma and urine of Anu was significantly ($P<0.001$) higher than control, Fes and AFes respectively.*
Conclusion: *Therefore, Ferrous sulphate restores kidney cytoarchitecture, reverses proteinuria and glycosuria in rats intoxicated with phenylhydrazine.*
Keywords: *Phenylhydrazine, renal cytoarchitechture, proteinuria, glycosuria Ferrous sulphate*

Date of submission: 20-08-2024 Date of acceptance: 30-08-2024

I. Introduction

PHZ belongs to the hydrazine family and is known to be one of the most potent carcinogens (1). It was first characterized by Hermann Emil Fischer in 1875 (2). The derivatives of PHZ were initially used as antipyretics but because they destroyed erythrocytes of patients they were used on; the use was stopped (3). PHZ increases reactive oxygen species (ROS) and lipid peroxidation and decreases glutathione (GSH); these effects are reversed by N-acetyl cysteine, a known ROS (4). PHZ generates ROS within both human and rat erythrocytes; no evidence for lipid peroxidation or phosphatidyl serine externalisation was detected (5, 6). ROS production was associated with extensive binding of oxidized and denatured haemoglobin to the membrane cytoskeleton. Thus, PHZ-induced haemolytic injury seems to be derived from oxidative alterations to red blood cell proteins rather than to membrane lipids (6). The damage on the red blood cell occurs on the membrane and this causes haematological alterations that cause the release of inflammatory mediators leading gradually to apoptosis (7). PHZ has been reported to cause lipid peroxidation in liver, kidney and spleen of mice (8). It induces oxidative damage to haemoglobin (9), membrane phospholipids and proteins in erythrocytes of humans (10,11). PHZ-induced haemolytic anaemia appears to result from oxidative damage to erythrocyte proteins rather than to interactions of membrane lipids (6). The PHZ-induced haemolytic anaemia occurs within 48h after lysis of erythrocytes (Berger, 2007). Apart from a decrease in red blood cells and haemoglobin concentrations PHZ intoxication has been reported to cause several other disorders as follows: derangement of electrolytes (12, 2), decrease in leucocyte, lymphocyte and thrombocyte counts, increase serum urea concentration and cause histopathological alterations of the kidneys of Wistar rats (13). The oxidative stress induced by PHZ has also

been reported to cause vascular dysfunction (14), a decrease in glomerular filtration rate (15,16). Albuminuria and proteinuria (17, 18) have also been reported to occur following haemolysis.

Proteinuria is a broad term used to describe protein in urine (19). The primary source of proteinuria is the kidney filter, and this is usually linked to acute kidney disease (20). Other causes of proteinuria are pregnancy, hypertension (21), primary tubular disease (22), myeloma (23). Proteinuria is a result of three different pathways, including: Glomerular dysfunction, Tubulointerstitial disease, secretory proteinuria (24), overflow proteinuria (25). The inflammatory effect of renal cancer and the decline in function of the kidneys can lead to proteinuria (26). The high metabolic demand of the tubulointerstitial makes it particularly susceptible to injury because the inflammation and associated edema compromise renal blood flow, causing a decrease in glomerular filtration rate (GFR) (27).

Glycosuria which is the passage of glucose in urine with or without an increase in the levels of glucose in circulation (28). When glycosuria is accompanied with hyperglycaemia, it is referred to as diabetes mellitus (29). Without hyperglycemia, it is referred to as renal glycosuria. This happens when the ability of the tubule to reabsorb glucose is impaired, e.g. Fanconi syndrome with impairment in the absorption of phosphate, amino acids, or isolated glucosuria as an inherited disorder termed Familial Renal glucosuria (28). Kidneys play a significant role in maintaining glucose homeostasis and preventing an individual from developing hypoglyemia. The maintenance of glucose homeostasis by the kidney includes glucose reabsorption in the FCT, gluconeogenesis, and the clearance of important hormones such as insulin. In one of our studies, we observed that there was glucose in the urine of rats fed photoxidised as well as thermoxidised palm oil even though the levels of glucose in blood was normal (30). Sequel to that, we observed that oxidative stress arising from phenylhydrazine intoxication led to an increase in glucose concentration in urine (31). In a very recent study (16) we observed that the excretion of glucose seen in our previous study (29) may have been caused by the downregulation of SGLUT2 activity/expression.

Creatinine is a non-protein nitrogenous compound that is produced by the breakdown of creatinine in muscle. Creatinine is found in serum, plasma, and urine and is excreted by glomerular filtration at a constant rate and in the same concentration as in plasma. Creatinine is a more reliable indicator of renal function than BUN because it is less influenced by other factors such as diet and hydration (32). It is very normal for kidneys to excrete creatinine in urine. Higher than normal creatinine levels in circulation are indicative of an acute kidney disease, usually owing to inflammation (33).

Several studies have shown that oxidative stress affects the histology of our tissue including the kidneys. Osim et al (34) showed that there were different levels of damage to tissues caused by thermoxidized palm oil. Other authors have shown that there are disruptions in the functions of several other organs, (31, 30, 35) typical with oxidative stress. Our previous studies have shown that there was distortion of electrolytes (31), increased plasma creatinine levels as well as aldosterone when Wistar rats were exposed to oxidative stress. (36). Ferrous sulphate has been shown to restore red ell count in haemolytic anaemia. It has also been shown to restore electrolyte levels. Can it reverse proteinuria and restore the cytoarchitecture of the kidneys after phenylhydrazine intoxication? The aim of this study is therefore to find out the effect of ferrous sulphate on the renal architecture and some renal function indices (protein, glucose and creatine levels in plasma and urine) in rats that have been intoxicated by phenylhydrazine.

II. Materials And Methods

Chemicals and Laboratory Equipment Used

The following drugs and chemicals were used during this study: Dimethyl sulfoxide (DMSO), phenlydydrazine (PHZ), disinfectant (Dettol-and methylated spirit), chloroform, 0.1NHCL, normal dextrose saline, 200mg ferrous sulphate tablets, distilled water, creatinine and urea reagents and potassium assay kits (Teco diagnostics company).

Laboratory Instruments/Equipment

The following instruments/equipment were used during this work: disposable syringes (1,2,5 and 10mls), retort stand, EDTA (Ethylene diamine tetra acid), sample bottles, feeding tube/cannula, Whatman filter paper, surgical gloves, beakers (500mls), stirrer, stop watch, funnels, white cotton material, desiccators, plain glass, stainless tray, tissue paper, sterile cotton wool, weighing balance, metabolic cages, dissecting board, micro-haematocrit centrifuge, test tubes of various sizes, urine bottles, Eppendorf tubes, dissecting set, dissecting board, water bath, bucket centrifuge machine (B-Bran Scientific and Instrument Company, England), pipettes, light microscope (B-Bran Scientific and Instrument Company, England), slides and cover slips, electronic weighing balance

Experimental Animals and Their Management:

A total of sixteen (16) adult male albino Wistar rates weighing between 200 – 250g were used for this experiment. The animals were obtained from the rat colony of the animal house of Pharmacology Department, University of Calabar. The rats were handled in accordance with international principles guiding the use and handling of experimental animals. They were maintained on standard rat feed (growers feed) and tap water which was made available ad libitum. The rats were maintained at an ambient temperature between 28° – 30°C; humidity of 55 ± 5% and standard (natural) photoperiod of approximately 12 hours of light (06:30 hour – 18:30 hour) alternating with approximately 12 hours of darkness (18: 30 hour – 06:30 hour).

Experimental Design

The animals were allowed to acclimatize for one (1) week. Thereafter, the animals were randomly distributed into four (4) groups of four (4) rats kept in separate metabolic cages. Group 1: Normal control group: received normal rat chow + tap water + distilled water (at 10ml/kg body weight) Group: Hematinic (ferrous sulphate) group: were fed normal rat chow + tap water + ferrous sulphate (using an oral gavage at 75mg/kg bw) Group 3: anemic-treated group: Phenylhydrazine (PHZ) was administered intraperitoneally for two consecutive days to induce anemia in the rats at a dose of 40mg/kg bw. Subsequently, the rats received normal rat chow + tap water + ferrous sulphate at 75mg/kg bw Group 4: Anemic control group: was administered with Phenlyhydrazine (PHZ) intraperitoneal at a dose of 40mg/kg of bw + Normal rat chow + tap water + distilled water (as in group one). The experiment lasted for 15 days.

Collection of Blood Samples

At the end of the experimental period, the animals were starved for 24 hours, and the animals anaesthetised with 60mg/kg pentobarbital and sacrificed. Blood samples were collected by cardiac puncture into EDTA and heparinized sample bottles. Plasma was immediately separated by centrifugation (3000g for 10mins). The plasma so separated was put in Eppendorf tubes and stored in a freezer until when needed for the estimation of sodium and potassium.

Histopathological Studies

The excised kidneys were fixed in 105 formalin for 3 days in wide necked universal containers. The fixed tissues were grossed after adequate fixation. First, the macroscopic appearance of the kidney was observed. They were observed for consistency, irregularity and size. They were weighed with weighing balance. Their dimensions were also taken. After the macroscopic examination, selected areas of the kidneys were cut with scalpel blade and put in tissue cassettes. They were sent for processing.

Tissue Processing

The grossed tissues were processed manually. Dehydration was done in 70%, 90%, 95% for 2 hours each and then in absolute 1 and 2; 2 hours for the first and overnight for the second. Clearing was done in three tissues were embedded in an embedding centre in metal embedding mould. The embedded tissues were trimmed to re-surface the tissue and placed on an ice block. They were sectioned with attached to the slide with egg albumin and placed on a hot plate.

Staining

The tissue slide was stained with Ehrlish's Haematoxylin and Eosin staining method to demonstrate the general structure of the kidney tissues.

Principle: This method involves application of haemalum which is a complex from aluminium ion and haematin, which is an oxidation product of haematoxylin. Haemalum stains the nuclei of cells and few other cell inclusions such as keratohyalin granules. The nuclear staining is followed by counter-staining with an aqueous solution of eosin Y which stains other eosinophilic structures in various shades of red, pink and orange.

Measurement of Glucose, Protein and Creatinine in blood and urine

Glucose levels in blood and urine samples were measured in triplicate with a Beckman Glucose Analyzer II (Beckman, Fullerton, Calif. USA).

Blood and urine levels of total protein were determined using commercially available kits and an automatic biochemistry analyser (Mindray BS-800, Shenzhen, China).

The creatinine concentration in both urine and blood samples were determined by Jaffe's reaction method of Bonsnes and Toussky (37). It is based on the principle that creatinine reacts with alkaline picrate solution to give a red colour (Jaffes reaction). The production of this red colour is non-specific since other non-creatinine substances in the blood are known to give similar reaction. But the recovery of creatinine in an acid

filterate helps to minimise the reaction after 15 minutes standing at room temperature. The 15 minutes timing must be strictly adhered to since the non-creatinine reaction maximises after 15 minutes.

Statistical analysis

Data were presented as mean ± SEM, Experimental data were analysed using Analysis of variance (ANOVA) followed by a post HOC test (Tukey's test) to determine significant differences between means. The analysis was done with graph pad prism version 8.02 (263) statistical package. $P<0.05$ was accepted as statistically significant.

III. Results

The photomicrograph plates of the kidneys belonging to the control, Fes, AFes and Anu are shown below.

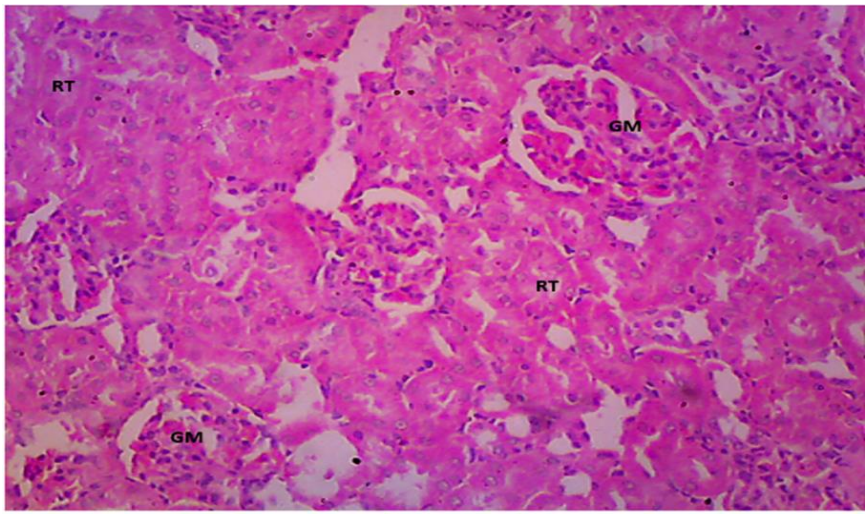

Plate 1: **Section of the kidney of a control animal. The histology is normal. There are visibly healthy renal tubules (RT) and normal glomeruli (GM) seen in image above.**

Plate 1 shows the photomicrograph of a section of a control animal. The histology is normal. Healthy renal tubular (RT) cells are visible. The glomeruli (GM) are clearly outlined and visible.

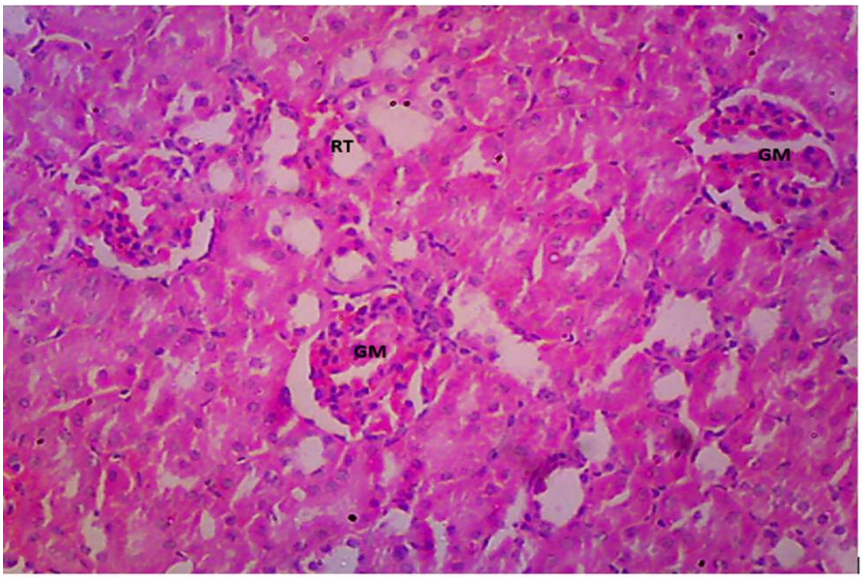

Plate 2: Section of a rat kidney from the Fes group. It shows very healthy cells with well-defined glomeruli (GM) and renal tubules (RT) clearly visible. Tissues look healthier than the control group.

Plate 2 shows the photomicrograph of an animal from the Fes group. It shows healthy well-defined glomeruli (GM) and renal tubules (RT). Tissues are looking as healthy as the control group.

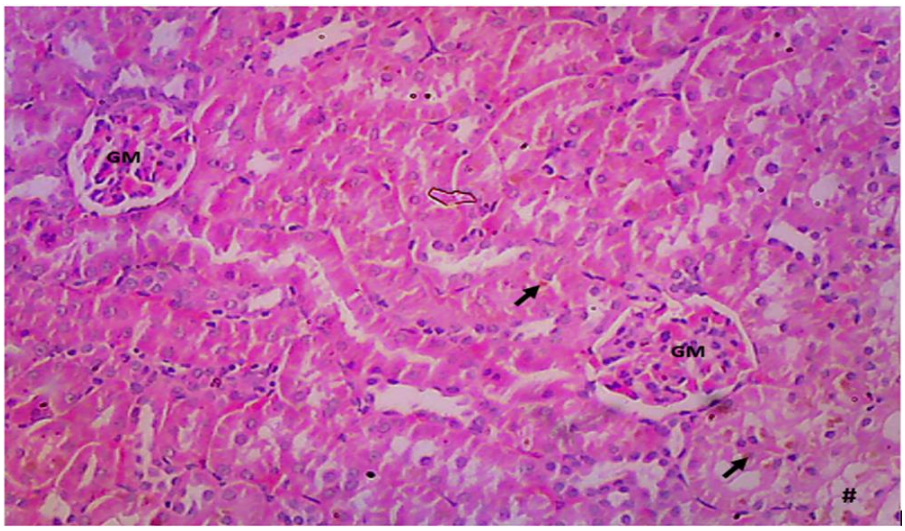

Plate 3: Section of a rat kidney from the AFes group showing normal glomeruli (GM) and renal tubules (RT), area of tissue degeneration (#) in an anemic untreated rat with light brown pigment (Arrow). (H&E) x100.

Plate 3 shows the photomicrograph of a kidney in recovery. This is the kidney of the anaemic treated group (AFes). There are a few areas of tissue degeneration (#) and some patches of inflammation (unlabelled arrow). The glomeruli (GM) are visible. The renal tubules, too, even though not labelled, are also visible. This shows that the tissues are in recovery.

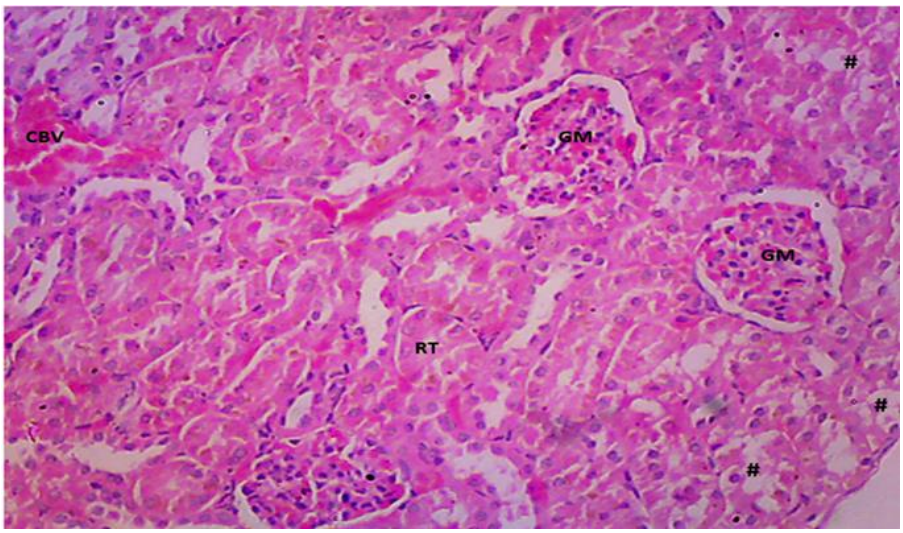

Plate 4: **Section of the rat kidney histology from the Anu showing normal glomeruli (GM), renal tubules (RT), congested blood vessels (CBV) in an anemic treated rat with an area of tissue degeneration (#). (H&E) x100**

Plate 4: shows the kidney of an anaemic untreated rat with kidney tissue clearly showing inflammation. There are areas where vessels are congested, showing inflammation (CBV) many more areas of tissue degeneration (#), some areas of inflammation around the tubules as well as the glomeruli, even though not labelled.

Table no 1: Comparison of the mean±SEM concentrations of glucose, protein and creatinine in plasma and urine of control, Fes, AFes and Anu groups of Wistar rats.

Parameters	Control	Fes	AFes	Anu
Glucose in plasma (mg/dl)	100±13.38	11.13±5.23ns	121±14.27ns	135±12.59ns
Glucose in urine (mg/dl)	0.00±0.00	0.00±0.00ns	0.12±0.014***, a	1.02±0.026***, b, c

Protein in plasma (mg/dl)	48.0±17.2	49.95±13.52ns	45.23±13.52ns	30.21±1.13ns
Protein in urine (mg/dl)	2.15±0.11	2.32±0.42	3.12±0.23	4.53±0.21***, b, c
Creatinine in plasma (mg/dl)	0.16±0.03	0.18±0.032ns	0.2±0.032ns	0.6±0.037***, a
Creatinine in urine (mg/dl)	59.73±3.21	58.38±2.53ns	63.28±3.27ns	83.52±3.52***, a

Ns, *** = not significant, P<0.001 vs control
a, b = P<0.01; P<0.001 vs Fes
s, c = P<0.01; P<0.001 vs AFes

From the table above, there was no significant difference in the plasma glucose concentration when all the test groups were compared with the control and each other. There was also no significant difference when the mean ± SEM concentration of glucose in the urine of Fes was compared with the control. However, on comparing the AFes and Anu groups with the control group, there was a significant (P<0.001; P<0.001 respectively) increase in glucose concentration of urine. There was also a significant (P<0.001) increase of glucose in urine concentration of AFes compared to Fes.

There was also no significant difference between all the groups when the mean ± SEM concentration of protein in circulation when all the groups were compared with each other. However, when the urine concentration in the Anu was compared with the control, Fes and AFes there was a significant (P<0.001; P<0.001; P<0.01) respectively increase. There was no significant difference in the concentration of protein in the urine control, Fes and AFes when they were compared to each other.

Creatinine concentration in the plasma of control, Fes and AFes were not significantly different from each other. However, the plasma creatinine concentration in the Anu was significantly (P<0.001) higher than control, Fes and AFes. The trend in the concentration of creatinine urine was the same with that of plasma as there was no significant difference when the control, Fes and AFes were compared with each other. The creatinine concentration in the urine of the Anu was significantly (P<0.001; P<0.01) higher than control and Fes.

IV. Discussion

The results from the present study shows that there was no significant difference in the plasma protein and glucose levels when all the groups were compared with each other. However, there was a significant increase in the protein and glucose urine levels in the Anu when compared with the control, Fes and AFes. Indicating that there was proteinuria and glycosuria in Anu. The creatinine levels in the plasma and urine of Anu were significantly higher than that of control, Fes and AFes. However, the extent to which the creatinine concentration in the urine of Anu animals rose compared to AFes and Fes was much more than it did in plasma. This implies that there was a decrease in creatinine clearance, hence GFR in Anu compared with AFes and Fes. The histological slides of the control and Fes groups looked normal. However, the histology of AFes showed marked recovery from inflammation compared to the control and Fes with fewer areas of vascular congestion and tissue degeneration.

As stated earlier, this study shows that there was glucosuria and proteinuria in Anu. Other studies have shown that proteinuria can arise from different sources such as: glomerular dysfunction, Tubulointerstitial disease, secretory proteinuria (24), overflow proteinuria (25). Previous studies have shown that there was proteinuria when animals were exposed to oxidative stress caused by chronic consumption of oxidised palm oil and phenylhydrazine (30,31). Therefore, in this study, the proteinuria that occurred may have been because of glomerular disease or tubulointerstitial disease which can result from oxidative stress. PHZ causes oxidative stress (24). Studies have also shown that oxidative stress can lead to inflammation and vice versa (38). On the other hand, some studies have shown that rapidly metabolising cells are prone to inflammation arising from lack of energy from lack of oxygen. The glomerular and tubuloepithelial cells are known to be highly metabolising cells, they are therefore prone to inflammation at the slightest lack of oxygen supply It is possible therefore, that the Anu glomerular and tubulointerstitial cells may have undergone inflammation. However, as shown on the plates and tabular results, ferrous sulphate led to a recovery from the proteinuria. Ferrous sulphate is known as the most effective hematinic recommended for haemolytic anaemia. It is obvious from the results obtained in this study that PHZ may affect other tissues apart from red blood cells indirectly. For glucose or proteins to appear in urine, there must have been inflammation of some sort to the kidneys. It is common knowledge that Ischemia can also lead to inflammation. In the case of haemolytic anaemia resulting from PHZ poisoning, there is a limitation to oxygen supply to the tissue. The tissue may have become Ischemic, and inflammation may have set in causing the glomerular filter to allow larger than normal amounts of protein to be filtered through.

From the photomicrographs of Anu animals as shown in the results, the picture is even clearer because there were areas of redness signifying inflammation and other areas of tissue degeneration, especially around the

tubules. This clearly shows that the tissues may have suffered Ischemia as tissue can barely survive for long amidst ischemia. In the AFes, we see remarkable recovery. This shows that the degeneration may have originated primarily from limited blood supply to the tissue. It also shows that tissue recovery may have led to the restoration of protein levels and reversal of proteinuria.

As for the glycosuria seen in this study, it was shown in our previous study (16) that oxidative stress has adverse effects on the main glucose transporter in the nephron (SGLUT2). It confirms the fact that there may have been tubular interstitial inflammation accounted for by the are small patches of vascular congestion in the photomicrographs of the AFes and Anu. To support the above, it has been shown that the tubular interstitial cells are susceptible to injury because of their high metabolic demands (27). Since SGLUT2 is located on the tubular epithelial cells, it is possible that the injury which may have occurred may have affected their structure leading to a reduction in activity leading to low glucose reabsorption to cause renal glycosuria. The difference between this study and our previous studies (30,16), is that in our previous studies, there was chronic exposure to oxidized palm oil whereas in this study, the stressor was phenylhydrazine. Another difference is that in this study, the exposure to the stressor (Phenylhydrazine) was acute not chronic. This may explain in part why within a short period of two weeks, the cytoarchitecture of the kidneys may have recovered enough to restore the functionality of this transport protein back to near normal in the AFes group.

Creatinine levels are usually lower in circulation because it is a waste product which is completely excreted in urine and not reabsorbed. This is the reason why it is often used to estimate glomerular filtration rate. Higher than normal creatinine levels in circulation are indicative of either acute kidney injury (AKJ) or chronic kidney disease. The increase in creatinine levels seen in the Anu may have been because of acute kidney injury arising from the temporary shortage of oxygen supply which may have led to Ischemia. However, in AFes, there was no significant difference between the creatinine levels in this group compared with the control.

This study shows that the damage to tissue and derangement in renal function indices may have been secondary to Ischemia. The Ischemia may have arisen from shortage of oxygen supply. When there is anaemia, especially haemolytic or iron deficiency anaemia, the kidney tissues (as well as other highly metabolising tissue elsewhere in the body) can become injured leading to acute kidney disease (39). Diseased kidneys cannot stimulate the kidneys to produce erythropoietin, and this can worsen the anaemia (40).

A closer look at AFes and Anu shows that the inflammation to the kidney was more serious around the glomeruli and the tubules, which confirm the study by (41, 27), who stated that these tissues are more prone to inflammation owing to their highly metabolising cells.

Iron supplementation through various routes such as oral or IV and iron fortification of foods can help manage and treat iron deficiency (42). Now what does iron supplementation do? It helps replace the lost iron stores to encourage erythropoiesis and facilitate oxygen transportation throughout the body, to reverse whatever defect the oxygen shortage to tissue may have caused.

V. Conclusion

From this study we conclude that: PHZ initiated haemolytic anaemia can lead to inflammation of the kidney glomerular and tubular interstitial cells to cause proteinuria, glycosuria and a significant reduction in glomerular filtration rate.

Ferrous sulphate restores the tissues to normal indirectly though, through restoration of the red cell count to normal, hence ensuring adequate oxygen supply to highly/rapidly metabolising renal glomerular and tubular interstitial tissue.