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**Original Research Article**

# Ferrous Sulfate Reduces the Phenylhydrazine Induced Negative Correlation between Aldosterone Concentration and Creatinine Clearance (GFR) in Wistar Rats

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## Abstract

The aim of this study is to find out the effect of phenylhydrazine on creatinine clearance, hence GFR and the relationship between GFR and aldosterone. 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 - Anemic -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 Phenlyhydrazine (PHZ) intraperitoneally at a dose of 40mg/kg of bw + normal rat chow + tap water (as in group one). After 15 days, blood and urine samples were collected into sterile sample bottles for analysis. There was a significant ($P<0.01$, $P<0.01$, $P<0.05$) increase in aldosterone levels between Anu, control, Fes and AFes respectively. There was a significant ($P<0.001$) decrease in control compared with Anu. There was also a significant ($P<0.01$, $P<0.001$) decrease in Fes with AFes and Anu. Anu creatinine clearance was also significantly ($P<0.001$) lower than AFes. Phenylhydrazine intoxication led to a reduction in creatinine clearance and an increase in aldosterone levels, confirming a negative correlation ($r= 0.9956$, $P<0.01$) between aldosterone and creatinine clearance. Also, ferrous sulphate tends to reduce the extent to which aldosterone levels increased hence narrowing the margine and or reducing the significance of the correlation.

**Keywords**: Creatinine Clearance, Aldosterone, Phenylhydrazine, Glomerular Filtration Rate, Oxidative Stress, Kidney.



## INTRODUCTION

Phenyl hydrazine (PHZ) a potent chemical causes toxicity on various tissues at various levels. Its administration mainly causes hemotoxicity which leads to hemolytic anemia. In mammals, PHZ-induced anemia increases iron absorption in the spleen, liver and duodenum; after-which Fe metabolism is altered. Administration of PHZ also interferes with the binding of Erythropoietin with its receptor-EPOR. PHZ is toxic by single intraperitoneal administration and is expected to be toxic by the inhalation and dermal routes (Berger, 2007). This chemical has potential for skin and eye irritation in human (Berger, 2007). Exposure to phenylhydrazine may cause damage to red blood cells, potentially resulting in anaemia and consequential secondary involvement of other tissue, such as the spleen and liver (Moreau, 2012)

The accompanying oxidation of phenylhydrazine leads to the formation of several products including benzene, nitrogen, hydrogen peroxides, superoxide anion and the phenyl-radical (Banerjee *et al*, 2020). 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). Many researchers, in an attempt to study haemolytic anaemia and its associated

effects, use the chemical, phenylhydrazine (PHZ), to induce anaemia. 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.

Anaemia is of different types depending on the causative factor. For 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). Iron therapy is contraindicated in most hemolytic anemias. However, iron therapy is indicated for patients with severe intravascular hemolysis in which persistent hemoglobinuria has caused substantial iron loss (Baldwin, 2023). Ferrous sulfate is the most common and inexpensive form of iron used.

Glomerular filtration rate (GFR) describes the flow rate of filtered fluid through the kidney. Creatinine clearance rate (CCr or CrCl) is the volume of blood plasma that is cleared of creatinine per unit time and is a useful measure for approximating the GFR.

Aldosterone is the main mineralocorticoid synthesized by the adrenal gland and has an essential function in sodium and water homeostasis and urinary excretion of potassium (Roldan *et al*., 2010). Studies have shown that aldosterone plays an important pathogenic role in vascular remodeling in renal disease (Yoshimoto and Hirata, 2007). In advanced stages of renal failure, aldosterone values increase significantly as glomerular filtration decreases (Wenzel, 2008). In a 2010 study by Roldan *et al*, an interesting relationship was found between aldosterone and glomerular filtration: the higher the aldosterone level in hypertensive patients with normal renal function, the lower the renal fitration values. Since oxidative stress is known to cause tissue damage that can cause damage to both cardiovascular and renal tissue leading to hypertension and kidney failure eventually. Is tye oxidative stress induced by phenylhydrazine intoxication enough to cause increased aldosterone and reduced creatinine clearance? If yes, can ferrous sulphate reverse this; since it is one of the most trusteed supplements administered for hemolytic anaemia? The aim of this study is to find out whether phenylhydrazine which is a known toxicant can produce the above results. How will this affect water output and renal handling of sodium and potassim handling.

## MATERIALS AND METHODS

### Drugs and Chemicals Used

The following drugs and chemicals were used during this work: Dimethyl sulfoxide (DMSO), phenlyhydrazine (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 the course of this work: disposable syringes (1,2,5 and 10mls), retort stand, EDTA (Ethylene diamine tetra acetic 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, Sahli's-apparatus, tissue paper, sterile cotton wool, weighing balance, metabolic cages, dissecting board, micro-haematocrit centrifuge, test tubes of various sizes, urine bottles, 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 rats 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. The rats 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 – 30oC, 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. The experiment lasted for 15 days.

**Group 1**: Normal control group: received normal rat chow + tap water + distilled water (at 10ml/kg body weight)

**Group 2:**

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)

Measurement of urine volume Each of the metabolic cages was unmounted daily and the 24 hours urine output of each rat was measured by collecting the urine in the urine collecting chamber with a 10ml syringe and reading off. All urine volumes were recorded in mls.

**Collection of Blood Samples**

At the end of the experimental period, the animals were starved for 24 hours and were made unconscious using chloroform anesthesia soaked in cotton wool and blood samples from each rat were collected via cardiac puncture technique (Ohwada, 1986) into EDTA and heparinized sample bottles. Plasma was immediately separated by centrifugation (300 g for 10 mins). The plasma so separated was put in Eppendorf tubes and stored in a freezer until when needed for the estimation of sodium and potassium.

**Determination of Sodium and Potassium in Plasma**

The concentration of potassium and sodium in diluted plasma and urine was done using flame photometry as described by Saud *et al*., (2020).

Principle; the solution, i.e plasma/urine containing sodium and potassium was sprayed as fine droplets. Due to the heat of the flame, the droplets dried up leaving a fine residue of salt. This fine residue got converted into neutral atoms.

Due to the thermal energy of the flame, the atoms got excited and thereafter returned to ground state. During their return to ground state, excited atoms emitted radiation of specific wavelength. This wavelength emitted is specific for every element. That for potassium was 766nm and emitted a violet colour while that for sodium was 590nm and emitted a yellow colour.

**Determination of Creatinine**

The Creatinine concentration in both urine and serum samples were determined by Jaffe's reaction method of Bonsnes and Toussky (Chromv *et al*., 2008).

**Principle:**

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.

**Creatinine Clearance**

Creatinine clearance in ml/min was calculated by multiplying urine creatinine concentration by the urine volume and dividing the result by the plasma creatinine concentration value and then multiplying the total result by 1440 (60 mins x 24hours).i.e: (Uc . V/ Pc) x 1440

**Where;**

Uc = Urine creatinine concentration.
V = 24hours Urine volume
Pc = Plasma creatinine concentration.

Creatinine clearance was calculated on the last day with the value of urine creatinine concentration and urine volume with the serum creatinine concentration value on the 15th day

**Statistical Analysis**

Data were presented as mean ± SEM. Experimental data were analysed using Analysis of variance (ANOVA) followed by a post HOC test (Least square difference (LSD (test) to determine significant differences between means. The analysis was done with an SPSS 18 statistical package. $P<0.05$ was accepted as statistically significant.

## RESULTS AND DISCUSSION

**Table 1: comparison of the mean ± SEM of K+, Na+ in blood and urine and the urine volume in the control, Fes, AFes and Anu**

| PARAMETER | CONTROL | Fes | AFes | Anu |
|---|---|---|---|---|
| $K^+$ in plasma (Meq/L) | 5.37±0.04 | 5.27±0.045 $^{ns}$ | 6.75±0.06***,a | 7.62±0.03***,a,b |
| $K^+$ in urine(Meq/L) | 22±0.62 | 21±0.51 $^{ns}$ | 22±0.76$^{ns}$ | 20±0.58 $^{ns,c,d}$ |
| $Na^+$ in plasma (Meq/L) | 132±0.08 | 134.5±0.95 $^{ns}$ | 133.25±0.47$^{ns,r}$ | 136.72±0.85 $^{**,s,t}$ |
| $Na^+$ in urine (Meq/L) | 99±1.52 | 102±1.02 $^{ns}$ | 110.6±1.81***,c, | 118.2±1.50***,a,b |
| Urine volume ml/day | 9.12±0.01 | 8.15±0.01*** | 8.02±0.025***,u | 6.97±0.032***,a,b |

*; **; *** = P<0.05; P<0.01; P<0.001 vs control
ns = not significant vs control
r, a, u, c =not significant; P<0.001; P< 0.01 and P<0.05 vs Fes
b, d, t = not significant; P<0.001; P<0.01 and P<0.05 vs AFes
s = not significant vs Anu

Table 1 shows the comparison of the mean ± SEM of $K^+$, $Na^+$ concentrations in blood and urine and the urine volume in the control, Fes, AFes and Anu.

There was no significant difference in plasma K+ concentration between Fes and control. However, there was a significant (P<0.001) increase in plasma K+ concentration in AFes and Anu were compared with the control and when Anu was compared with Fes and Afes (P<0.001 and P< 0.001 respectively). When the urine K+ concentrations of the control group was compared with Fes, Afes and Anu, there was no significant difference. There was also no significant difference when AFes was compared with Fes. However, when Anu was compared with Fes and AFes, there was a significant (P<0.05 and P<0.01 respectively).

The comparison of plasma $Na^+$ concentration of the control and Fes and AFes showed no significant difference. There was however a significant (P<0.01) decrease when compared with Anu. As for the concentration of $Na^+$ in urine, there was no significant difference between the control and Fes. There was a significant (P<0.001. P<0.001 respectively) difference when AFes and Anu were compared with the control group. There was also no significant difference between AFes and Anu were compared with Fes. There was however a significant (P<0.05) increase in plasma sodium concentration when compared with AFes. There was no significant difference in urine Na+ concentration between the control and Fes while there was a significant (P< 0.001) difference when AFes and Anu were compared with the control group. The urine concentration of $Na^+$ was significantly (P<0.05) higher than Fes, while that of Anu was significantly (P< 0.001) higher than Fes and AFes.

The urine volume of AFes and Anu were significantly (P<0.001) lower than that of control, but there was no significant difference between control and Fes. AFes urine volume was significantly (P<0.01) lower than Fes while that of Anu was significantly (P<0.001) higher than Fes and AFes.

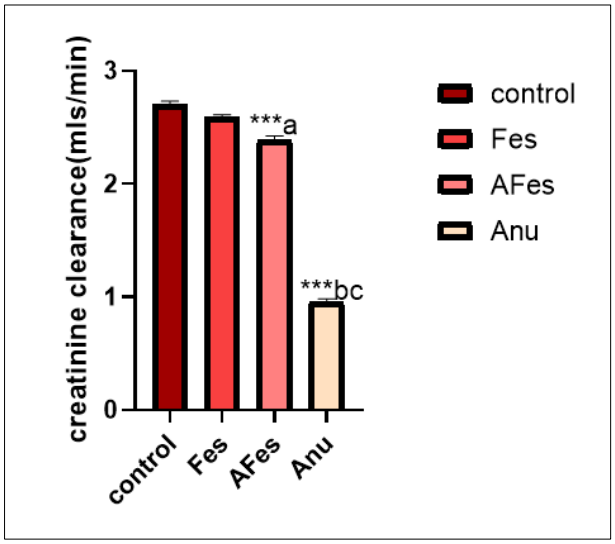


**Fig. 1**: **The comparison of mean ± SEM creatinine clearance between control, and Fes, AFes and Anu**

Fig 1 shows the comparison of mean ± SEM creatinine clearance between control (2.7±0.032) and Fes (2.59±0.021), AFes (2.39±0.035) and Anu (0.96±0.022). There was no significant difference between Fes and control while the creatinine clearance in AFes and Anu were significantly (P<0.001) lower than that of the control group. That of AFes was significantly (P<0.01) lower than Fes. The creatinine clearance of Anu was significantly (P<0.001) lower than Fes and AFes.

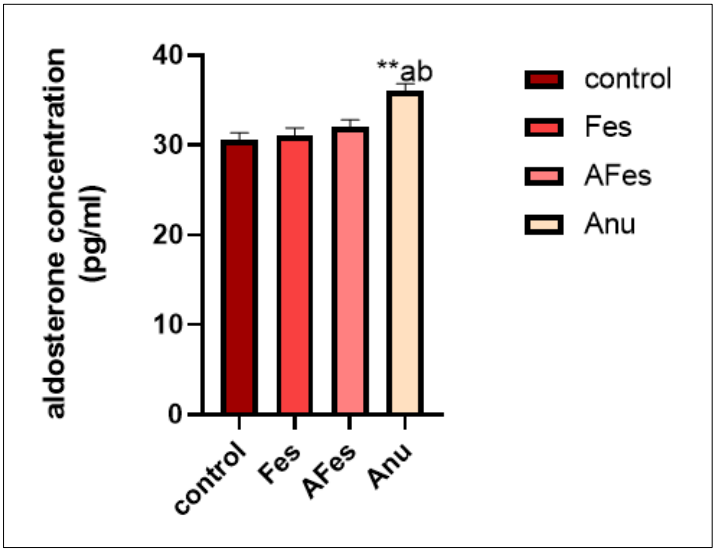


**Fig. 2**: **The comparison of mean ± SEM aldosterone levels between control, AFes, Fes and Anu**.

There was no significant difference between the control and Fes and AFes groups and between AFes and Fes. (Fig 2) There was however a significant (P<0.01; P<0.01 and P<0.05) difference when Anu was compared with the control, Fes and AFes respectively.

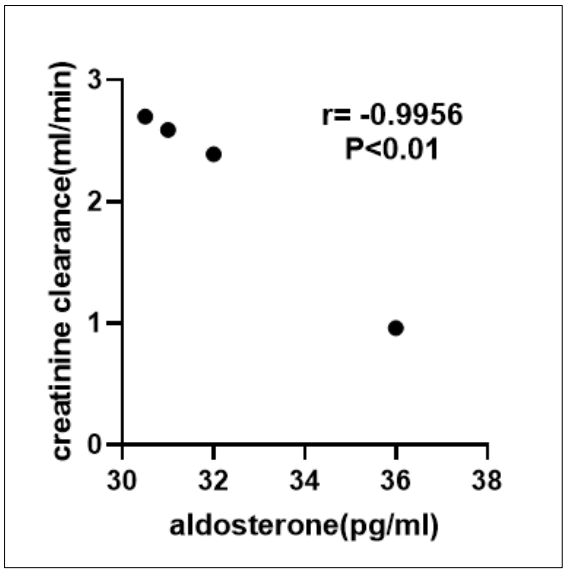


**Fig. 3: The Correlation analyses between the mean±SEMs of creatinine clearance and aldosterone in all the groups of animals.**

There was a strongly negative (r = -0.9956) and significant (P<0.01) correlation between creatinine clearance and aldosterone levels in plasma as shown in Fig3.

The findings in the present study show that glomerular filtration rate is significantly reduced in phenylhydrazine toxicity while aldosterone levels are significantly increased. This lead to distorted sodium and potassium levels with potassium as well as sodium levels rising in plasma. We also confirmed the negative correlation between aldosterone and creatinine clearance which is an indication of glomerular filtration rate as observed by Roldan *et al.*, (2010).

The reduction in glomerular filtration rate seen in this study is in line with previous studies by Haymann, *et al*., (2010). Oxidative stress has been linked with phenylhydrazine intoxication. It has also been established that oxidative stress which often results from an accumulation of ROS leads to damage to tissue and functionality of organs. The oxidative stress-linked reduction in creatinine clearance, hence GFR seen in this study, was expected.

Aldosterone levels were seen to also increase. This may have been because of the increased potassium levels. Hyperkalemia is known to be one of the most potent stimuli for aldosterone release (ref). Increased aldosterone also leads to an increase in excretion of potassium to balance osmotic as well as electrochemical gradient of plasma. In this study however, we see that the aldsoterone levels did not lower the potassium levels. This may have been because oxidative stress also causes cell lysis which may have led to the increase in an otherwise intracellular electrolyte level in plasma. Presumably, the aldosterone may get to reduce extracellular fluid/plasma levels of potassium once the effects of toxicant which in this case is phenylhydrazine is reversed or wears off. The aldosterone leads to an increase in sodium in circulation. The increased sodium levels in plasma seen in this study may have been because of the increased aldosterone levels in plasma.

Aldosterone levels were seen to correlate negatively with creatinine clearance. This even though is in line with previous studies (Roldan *et al*., 2010), it is still not clearly understood why. Now, when aldosterone levels rise, sodium which is a volume expander rises to increase the volume of extracellular fluid, which should increase blood pressure and glomerular filtration rate. In this study however, we see a decrease in filtration rate and even in urine production. It is possible that the glomeruli may have been inflamed too since this is one of the effects of oxidative stress (ref).

Previous studies had shown the phenylhydrazine led to the derangement of electrolytes (Beshel *et al*., 2019). In another study, it was shown that oxidative stress caused an increase in blood pressure with reduced glomerular filtration rate (Beshel *et al*., 2014). Wenzel, in 2008 observed that in advanced stages on renal failure, aldosterone levels increase significantly as glomerular filtration rate decrease. Therefore, oxidative stress may have led to kidney disease, causing decreased creatinine clearance and hence GFR, and an increase in aldosterone levels in this study. Even though blood pressure was not measured, there was a significant decrease in creatinine clearance which is an estimation of glomerular filtration rate, which confirms the results gotten previously by Rolden *et al*., (2010). The negative correlation between aldosterone and creatinine confirmed in this study is yet to be explained. We recommend further studies to unravel this.

When the results of all the groups were compared with each other, we observed that the results of the AFes group which were administered ferrous sulphate and phenylhydrazine showed some form of recovery towards normal. Ferrous sulphate as we have seen earlier, is the most appropriate hematinic supplement for hemolytic anemia. Hemolytic anaemia may have triggered some degree of oxidative stress in this study in addition to that which may have been caused by phenylhydrazine on its own. This actually makes a lot of sense because in hemolytic anemia (which is the type induced by phenylhydrazine), blood cells are lysed, they lose hemoglobin and the red blood cells lose their ability to carry oxygen. Tissues therefore go into oxidative stress because of lack of oxygen, and this may either affect the structure or the function of cells, especially in the kidneys. Introduction of ferrous sulphate begins the process of synthesis of the hemoglobin molecule endows red blood cells with the ability to carry oxygen.

When looking at the volume of urine excreted, it is also not strange to see that it reduced significantly in the untreated anaemic group (Anu). The creatinine clearance, hence glomerular filtration rate was significantly reduced. It therefore follows that since the glomerular filtration rate reduced, the volume of urine excreted will also be significantly reduced. This is in line with previous studies that have shown a significant decrease in both urine volume and flow rate because of consumption of oxidants.

## CONCLUSION

Phenylydrazine cause an increase in aldosterone and a decrease in creatinine clearance, hence glomerular filtration rate of wistar rats. The relation ship between aldosterone and creatinine clearance showed a significant negative correlation. The oxidative stress which occurred in this study may have been not only because of the derrivatives of phenylhydrazine, but also because of the shortage of oxygen arising from the hemolytic anemia and this is because the administration of ferrous sulphate to animals already exposed to phenyl hydrazine began to reverse the results seen in the Anu group.

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