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Obesity-induced renal injury is associated with oxidative stress via renal ischemia reperfusion injury

Christian Eseigbe Imafidon[a,b,*], Rufus Ojo Akomolafe[b]

[a] Department of Physiology, Faculty of Basic Medical and Health Sciences, Bowen University Iwo, Iwo, Osun State, Nigeria
[b] Department of Physiological Sciences, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria

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ABSTRACT

Background: Obesity and its increasing incidence is becoming a global concern. This condition is often associated with compromised health and reduced life expectancy. A proper understanding of the underlying mechanism(s) for its associated health risks can help to create better preventive and therapeutic measures against this life-threatening condition. This study investigated the relationship between obesity, oxidative stress and renal function.

Method: Twenty Wistar rats were divided into control and obese groups, consisting of ten rats per group. In the obese group, *ad libitum* feeding on high fat diet (> 12% fat content) for 13 weeks was used to induce obesity (BMI > 0.68 g/cm^2) while the control group fed on normal fat diet (< 5% fat content) and had BMI below the obesity index before the rats were euthanized.

Result: The obese rats had significantly lowered plasma activities of NO, GSH and SOD while their TBARS level was significantly higher than the control ($p < 0.05$). Also, deleterious derangements in the plasma and urine cystatin C, creatinine and urea concentrations as well as significantly lowered cystatin C clearance, creatinine clearance and FEurea were observed in the obese groups. There was micrographic evidence of atrophied and shrunken glomerulus with tubular and interstitial vacuolations in the obese when compared with the control.

Conclusion: It was concluded that obesity-induced renal injury is associated with oxidative stress via renal ischemia reperfusion injury.

1. Introduction

Obesity, a condition characterized by excessive fat accumulation to the extent that it may impair health and reduce life expectancy, is an emerging world health problem with increasing incidence (Haslam and James, 2005; Rodríguezhernández et al., 2013). According to literature, obesity is a potent risk factor for the development of various health disorders including metabolic syndrome, hypertension, cardiovascular disease, diabetes mellitus as well as chronic kidney disease (Eknoyan, 2011). Obesity increases the risk of developing kidney disease by increasing the metabolic demands of the kidney (Holly, 2006); a condition that may increase future decline in life expectancy, if left unchecked. The increasing prevalence of obesity-induced kidney injury has been projected to grow by 40% across the globe in the next decade (Kovesdy et al., 2016).

A mismatch between calorie intake and energy expenditure has been identified as an important risk factor for the development of obesity (Astrup et al., 1994; Lissner and Heitmann, 1995). This makes diet of importance to the incidence of obesity and to its negative health consequences (Bianchini et al., 2002; Novelli et al., 2002). Practicing a lifestyle of increasing food consumption with a reduction of physical exercise can easily result in obesity (Rodríguezhernández et al., 2013). Effective studies on the consequences of obesity in humans have ethical limitation, hence experimental models are adopted for the scientific study of obesity. Both humans and experimental rodents tend to gain weight with high calorie intake. Therefore, rats are frequently used as experimental animal models for the study of obesity (Andre et al., 2008; Tiago et al., 2012).

The mechanisms by which obesity induces health disorders are poorly understood, hence there is need for more scientific exploration in this scope of research. New scientific findings or additional scientific information may help provide better preventive measures and therapeutic strategies against this life-threatening condition.

Oxidative stress is a condition that reflects an imbalance between free radicals (molecules that causes damage to biological systems) and systemic antioxidants (molecules that neutralizes free radicals and their

* Corresponding author. Renal Research Laboratory, Department of Physiology, Bowen University Iwo, Iwo, Osun State, Nigeria.
E-mail address: staywithchris@gmail.com (C.E. Imafidon).

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Table 1
Composition of experimental diet.

Ingredients	Quantity in NFD (Kg)	Quantity in HFD (Kg)
Maize white	13.00	1.00
Wheat bran	3.75	1.08
Fish meal (72%)	1.00	–
Soya- full fat	–	20.00
Soya bean meal	3.24	–
Groundnut cake	3.00	2.00
Salt	0.13	0.10
Vitamin C	0.13	0.10
Limestone	0.25	0.12
Di-calcium phosphate (DCP)	0.50	0.60
Total quantity (Kg)	25	25
Total fat content (%)	3.50	15.81
Percentage difference in fat content	351.71 (%) •About 5 times higher than normal	

NFD = normal fat diet; **HFD** = high fat diet; **-** = absent.
Note: In literature, it has been reported that a normal rat feed contains an estimated 4% of total fat content, with 12% of total fat content being reliable to induce obesity in Wistar rats (Brownlee et al., 2001; Malik and Sharmaa, 2011).

deleterious biological effects) (Valko et al., 2007). Oxidative stress has been reported to play a critical role in the pathogenesis of various health disorders (Brownlee, 2001). For instance, by directly affecting the walls of the vascular system, oxidative stress underlies the pathophysiology of atherosclerosis and hypertension (Shigetada et al., 2004). It also decreases the secretion of insulin from pancreatic beta cells as well as causes the impairment of glucose uptake by fat and muscles (Maddux, 2001; Shigetada et al., 2004). Furthermore, the consumption of high fat diets has been implicated in the generation of oxidative stress in obese condition (Khan et al., 2006). Nevertheless, there is dearth of literature on the relationship between obesity, oxidative stress and renal function. This study aims at bridging this gap in knowledge by providing a mechanistic approach to this scope of research.

2. Materials and methods

2.1. Experimental diet, drugs, biochemical kits and metabolic cages

The experimental diet was formulated (and its automated proximate analysis determined) by ACE Feeds Company, Osogbo, Osun State, Nigeria (Table 1).

Pilocarpine was procured from Boehringer Ingelheim manufacturing company (Germany) while ketamine injection was procured from Arevi Pharma (Germany).

Standard biochemical kits were purchased from Randox Laboratories Limited (United Kingdom) and Quantikine ELISA (China).

Metabolic cages were fabricated by Central Technology Laboratory and Workshops (CTLW), Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

2.2. Animal management and experimental design

All experimental protocols were in strict compliance with the guidelines for animal research, as contained in the NIH guidelines for the care and use of laboratory animals (Guide for the Care and Use of Laboratory Animals) and approved by local Institutional Research Committee.

Twenty male Wistar rats of about 9–10 weeks old, weighing 135–150 g, were divided into two groups of 10 rats each as follows; **Control group** which was allowed 13 weeks feeding on normal fat diet (NFD) *ad libitum* after which they were sacrificed and **obese group** which was allowed 13 weeks feeding on high fat diet (HFD) *ad libitum* before they were sacrificed (Fig. 1). The experiment was terminated when rats in the obese group became obese, as defined by a body mass

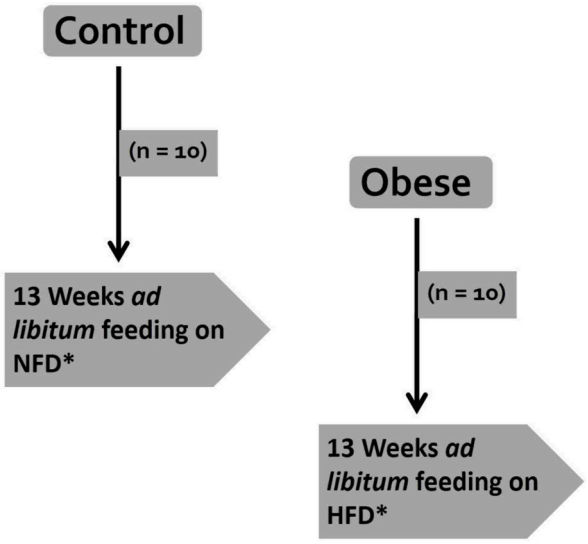

Fig. 1. Experimental Protocol. **n** = Number of rats in the group; **NFD** = Normal fat diet (< 5% fat); **HFD** = High fat diet (> 12% fat); * = point at which the rats were sacrificed.

Table 2
Body Mass Index (BMI) of the rats at the point of sacrifice.

	Control	Obese
BMI (g/cm^2)	0.58 ± 0.01	0.72 ± 0.01 $^{\alpha}$

Each value represents mean ± S.E.M at $p < 0.05$.
α = significant difference when compared with the control.
Note.
Obesity in Wistar rats was defined by a BMI greater than 0.68 g/cm^2 (Novelli et al., 2007).

index (BMI) > 0.68 g/cm^2 (Bernardis, 1970; Novelli et al., 2007) while the control had BMI that was less than the obesity index (Table 2). At the end of 13 weeks, 24 h urine samples were collected from the rats inside metabolic cages. Thereafter, their blood samples were collected by cardiac puncture into separate lithium heparinized bottles under ketamine anesthesia (60 mg/kg i.m). The blood samples were centrifuged at 4000 rpm for 15 min using a cold centrifuge (Centrium Scientific, model 8881) at −4 °C. The plasma obtained was decanted into separate plain bottles for biochemical assays. Also, the kidney of each rat was carefully excised and fixed in 10% formal saline solution for histological assessment.

2.3. Assessment of plasma activities of nitric oxide (NO), reduced glutathione (GSH), superoxide dismutase (SOD) and thiobarbituric acid reacting substances (TBARS) of the rats

The plasma activity of nitric oxide (NO) was determined using standard laboratory protocol as described by Grisham and co-workers (Grisham et al., 1996). This is described below;

Into separate Eppendorf tubes was added 100 μL of each sample followed by the addition of 0.1 ml of 1% sulphanilamide in 5% phosphoric acid. The resulting mixture was allowed to incubate for 10 min after which 0.1 ml of 0.1% NED was added and, thereafter, incubated for 10 min at 60 °C. The absorbance of the chromophore that was formed was read at a wavelength of 546 nm. A standard curve was prepared using sodium nitrite (100 μM) and the amount of NO in each sample was extrapolated from the standard curve.

The plasma level of reduced glutathione (GSH) was determined according to the method of (Beutler et al., 1963) as described below;

To 1 ml of the sample, 0.5 ml of Ellman's reagent (10 mM) and 2 ml

of phosphate buffer (0.2 M, pH = 8.0) were added. The yellow precipitate developed was read at 412 nm against a blank containing 3.5 ml of phosphate buffer. Series of standard were treated similarly and the amount of GSH was expressed in μg/ mg protein.

The plasma activity of superoxide dismutase (SOD) was determined by the method of McCord and Fridovich (McCord and Fridovich, 1969) and described as follows;

To 200 μL of each sample, 2.5 ml of 75 mM of tris–HCl buffer (pH 8.2), 30 mM EDTA and 300 μL of 2 mM of pyrogallol were added. An increase in absorbance was recorded at a wavelength of 420 nm for 3 min using a spectrophotometer. A unit of enzyme activity represented 50% inhibition of the rate of auto-oxidation of pyrogallol as determined by the change in absorbance per min at 420 nm. The activity of SOD was expressed as units/mg protein as defined by the equation below;

$$\text{Increase in absorbance per minutes} = \frac{A_3 - A_0}{2.5}$$

where A_0 = absorbance after 30 s; A_3 = absorbance after 150 s.

The plasma level of thiobarbituric acid reacting substances (TBARS) was determined by the method of Ohkawa and co-workers (Ohkawa et al., 1979) as described below;

To 0.5 ml of the sample was added 0.5 ml of phosphate buffer (0.1 M, pH 8.0) and 0.5 ml of 24% TCA. The resulting mixture was incubated at room temperature for 10 min, followed by centrifugation at 2000 rpm for 20 min. To 1 ml of the supernatant was added 0.25 ml of 0.33% TBA in 20% acetic acid and the resulting mixture was boiled at 95 °C for 1 h. The resulting pink colouring product was cooled and the absorbance was read at 532 nm.

2.4. Determination of plasma and urine concentrations of cystatin C, creatinine and urea of the rats

Plasma and urine cystatin C concentrations were determined by enzyme-linked immuno-sorbent assay (ELISA) using Quantikine ELISA kit (China). The procedure was carried out according to the manufacturer's instruction.

Plasma and urine concentrations of creatinine and urea were determined using Randox laboratory kits (Randox, United Kingdom). The procedures were carried out according to the manufacturer's instructions.

2.5. Determination of BMI, cystatin C clearance, creatinine clearance and fractional excretion of urea

The body mass index (BMI) of the rats was determined as follows; (Bernardis, 1970; Novelli et al., 2007).

$$\text{Body mass index } (g/cm^2) = \frac{\text{Body weight(g)}}{\text{Nose}-\text{to}-\text{anus length}^2(cm^2)}$$

Both cystatin C and creatinine (renal) clearance was determined using standard conventional formula as shown below;

$$\text{Renal clearance(ml/min)} = \frac{U_{\#}V}{P_{\#}}$$

where # = cystatin C or creatinine; $U_{\#}$ = urine concentration of cystatin C or creatinine; $P_{\#}$ = plasma concentration of cystatin C or creatinine; V = urine flow rate = amount of urine (ml)/time (mins).

The fractional excretion of urea (FEurea) was determined as follows; (Carvounis et al., 2002).

$$\text{FEurea}(\%) = \frac{U_{urea}\, x\, P_{cr}}{P_{urea} x\, U_{cr}} x\ 100$$

where U_{urea} = urine urea concentration; P_{urea} = plasma urea concentration; P_{Cr} = plasma creatinine concentration; U_{Cr} = urine creatinine concentration.

Table 3
Effects of obesity on plasma and urine concentrations of cystatin C, creatinine and urea in Wistar rats.

Cystatin C (mg/l)	Plasma concentration	Urine concentration
Control	4.84 ± 0.09	74.07 ± 0.87
Obese	5.68 ± 0.11 $^{\alpha}$	63.95 ± 0.50 $^{\alpha}$
Creatinine (mg/dl)		
Control	0.71 ± 0.01	14.70 ± 0.16
Obese	0.85 ± 0.02 $^{\alpha}$	12.90 ± 0.10 $^{\alpha}$
Urea (mg/dl)		
Control	37.67 ± 0.53	492.20 ± 4.60
Obese	43.49 ± 0.88 $^{\alpha}$	344.70 ± 7.10 $^{\alpha}$

Each value represents mean ± S.E.M at $p < 0.05$.
α = significant difference when compared with the control.

Table 4
Effects of obesity on micrographic kidney injury score of Wistar rats.

	Control	Obese
Injury score (per $1.80 \times 10^5 m^2$)	0.50 ± 0.29	3.75 ± 0.63 $^{\alpha}$

Each value represents mean ± S.E.M at $p < 0.05$.
α = significant difference when compared with the control.

2.6. Histological examination and assessment of micrographic kidney injury score

Histological examination of the kidney was by Hematoxylin – Eosin (H & E) staining technique. Thereafter, representative photomicrograph (at ×400 magnification) was imported to "Image J" software for kidney injury score as follows;

Each imported representative slide, with an area of $1.08 \times 10^6 m^2$, where subdivided into four complete squares, each with an area of $1.80 \times 10^5 m^2$, using grid lines. Thereafter, evidence of tubular and interstitial vacuolation as well as atrophied and shrunken glomerulus were counted per grid (scored). The number(s) obtained per grid was summed up and expressed as mean ± standard error of mean (n = 4; where n = number of grid). The value obtained represented the kidney injury score (Table 4).

2.7. Statistical analysis

Data were expressed as mean ± Standard Error of Mean using student's t-test and the level of significance was set at $p < 0.05$. The data collected were analyzed using graph pad prism (Graph Pad Software Inc., version 5.03, CA, USA) statistical package.

3. Results

High fat diet (consisting of > 12% of total fat content) (Table 1) was used to induce obesity in the Wistar rats.

At the point of sacrifice, the obese group had a significantly higher BMI when compared with the control ($p < 0.05$) while the control group had a BMI below the obesity index (Table 2).

3.1. Effects of obesity on the activities of plasma NO (μM) and plasma TBARS (nmol/ mg protein) in Wistar rats

The plasma NO activity was significantly lower in the obese group (0.92 ± 0.04) when compared with the control (1.10 ± 0.30) ($t = 3.779$; $p = 0.0014$; $F = 1.699$). However, the activity of plasma TBARS was significantly higher in the obese (1.47 ± 0.04) when compared with the control (1.21 ± 0.04) ($t = 4.128$; $p = 0.0006$; $F = 1.027$) (Fig. 2).

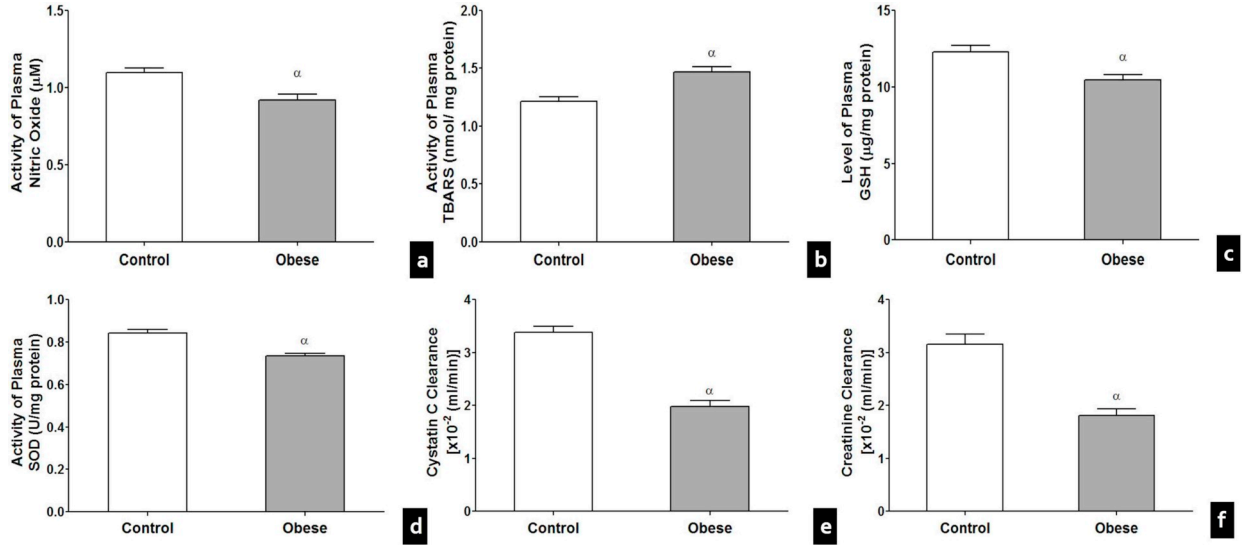

Fig. 2. Effects of obesity on the (a.) activity of plasma nitric oxide (NO); (b.) activity of plasma thiobarbituric acid reacting substances (TBARS); (c.) level of plasma reduced glutathione (GSH); (d.) activity of plasma superoxide dismutase (SOD); (e.) cystatin C clearance; and (f.) creatinine clearance of Wistar rats Each bar represents mean ± Standard Error of Mean (S.E.M) at $p < 0.05$. α = significant difference when compared with the control.

3.2. Effects of obesity on the level of plasma GSH (μg/ mg protein) and the activity of plasma SOD (U/mg protein) in Wistar rats

There was a significantly lower plasma GSH level in the obese group (10.46 ± 0.32) when compared with the control (12.27 ± 0.43) ($t = 3.361$; $p = 0.0035$; $F = 1.794$). Also, the activity of plasma SOD was significantly lower in the obese group (0.74 ± 0.01) when compared with the control (0.84 ± 0.02) ($t = 5.118$; $p = 0.0001$; $F = 3.923$) (Fig. 2).

3.3. Effects of obesity on plasma and urine concentrations of cystatin C (mg/l), creatinine (mg/dl) and urea (mg/dl) in Wistar rats

The plasma concentrations of cystatin C, creatinine and urea were significantly higher in the obese group when compared with the control ($p < 0.05$). However, the urine concentrations of cystatin C, creatinine and urea were significantly lower in the obese group when compared with the control ($p < 0.05$) (Table 3).

3.4. Effects of obesity on cystatin C clearance [$\times 10^{-2}$ (ml/min)], creatinine clearance [$\times 10^{-2}$ (ml/min)] and fractional excretion of urea (%) in Wistar rats

The obese group had a significantly lower cystatin clearance (1.98 ± 0.12) when compared with the control (3.39 ± 0.11) ($t = 9.013$; $p = 0.0001$; $F = 1.218$). Also, the creatinine clearance of the obese group was significantly lower (1.81 ± 0.12) than that of the control (3.16 ± 0.19) ($t = 6.067$; $p = 0.0001$; $F = 2.207$) (Fig. 2).

The fractional excretion of urea was significantly lower in the obese group (48.32 ± 2.14) when compared with the control (56.08 ± 1.47) ($t = 2.989$; $p = 0.0079$; $F = 2.127$) (Fig. 3).

3.5. Histological effects and kidney injury score

3.5.1. Histological effects of obesity on the kidney of Wistar rats

Micrographic evidence showed that obesity is associated with atrophied and shrunken glomeruli as well as vacuolation of the distal tubule and medullary interstitium (Fig. 4). This was in contrast to that of the control which showed evidence of apparently normal glomerulus with normal-appearing proximal and distal tubules (Fig. 4).

3.5.2. Effects of obesity on micrographic kidney injury score of Wistar rats

Kidney injury score using image J software showed that obesity was associated with a significantly higher area per square meter of kidney injury (characterized by tubular and interstitial vacuolation as well as distorted glomerulus) when compared with the control ($p = 0.0033$) (Table 4).

4. Discussion

This study demonstrated that the consumption of high fat diet (> 12% total fat content) for 13 weeks (*ad libitum*) induced obesity in Wistar rats and that this condition was associated with oxidative stress and renal dysfunction through renal ischemia reperfusion injury.

Nitric oxide (NO), an endogenous molecule that is exclusively synthesized from its L-arginine precursor and catalyzed by nitric oxide synthase (NOS), is a potent vasodilatory chemokine (Sharma, 2004). However, emerging evidence demonstrates its relevance in vascular biology in terms of its anti-inflammatory, anti-proliferative, anti-thrombotic and potent antioxidant effects (Rezaei and Mohhamad, 2018; Sharma, 2004). Furthermore, a balance in NO homeostasis has been reported to be essential for normal kidney function due to its critical role in biological mechanisms such as tubulo-glomerular feedback, regulation of glomerular and medullary hemodynamics, rennin release as well as the regulation of extracellular fluid (Oritz and Garvin, 2002; Rezaei and Mohhamad, 2018). Since NO is a potent vasodilator, which allows for increased perfusion, the implication of the obesity-related decline in circulating NO level on the renal system is reduced renal perfusion. The reduced renal perfusion was corroborated by a significantly lowered FEurea in the obese group. Decreasing FEurea level is a reflection of pre-renal effects resulting from reduced renal perfusion (Carlos et al., 2015; Christos et al., 2002). Although obesity is often associated with increased perfusion due to increase in metabolic demands, this study suggests that; “in the pathophysiology of obesity-related kidney injury, there is an initial increase in renal perfusion up to a threshold that is accompanied by a decline in renal perfusion”. The decline may be associated with a reduction in the concentration and or activity of vasodilatory chemokines such as NO.

Ischemia reperfusion injury, also called reperfusion injury, refers to the cell damage resulting from the return of blood supply after a period of ischemia (inadequate blood supply to an organ or part of the body) (Christopher and Homer-Vanniasinkham, 2003). Reperfusion injury

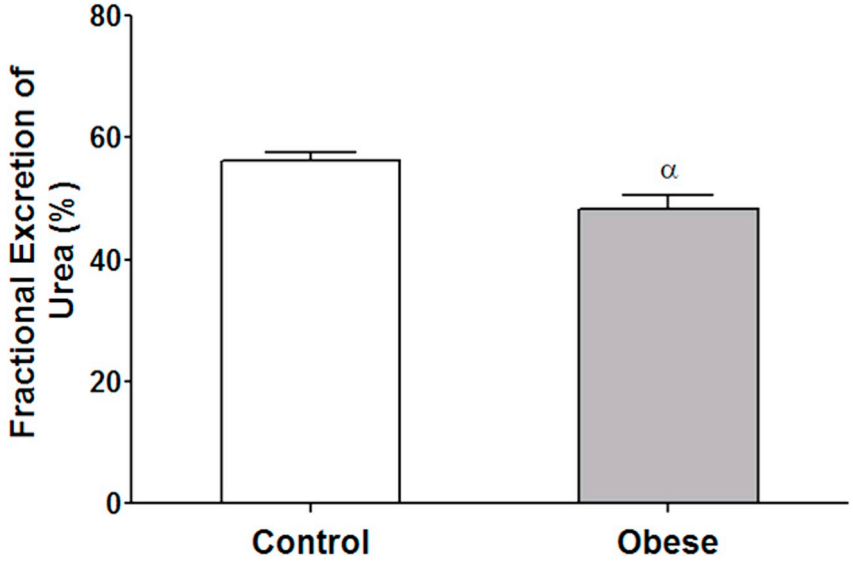

Fig. 3. Effects of obesity on fractional excretion of urea in Wistar rats. Each bar represents mean ± S.E.M at $p < 0.05$. α = significant difference when compared with the control.

causes excessive generation of reactive oxygen species (ROS) due to damage of mitochondrial complexes (Chen et al., 2008). The reduced renal perfusion, as recorded in this study, suggests a possible reperfusion injury. It was observed that the renal ischemia reperfusion injury that is associated with obesity involves interplay between circulating NO level and the ability of the kidney to auto regulate itself. The kidney is a vital organ that can auto regulate itself (maintain a fairly constant blood supply despite variations in arterial supply) (Stuart, 2011). Therefore, reduced circulating NO level must coincide with compromised renal auto regulation capacity for renal ischemia reperfusion injury to occur. The reperfusion injury provides an explanation for the reduced level of GSH (non-enzymatic antioxidant index) and SOD (enzymatic antioxidant index) in the obese group. It is an indication of excessive ROS generation beyond systemic antioxidant-scavenging capacity, hence obesity is associated with oxidative stress. Although this study provides a mechanistic view of oxidative stress that is associated with obesity-induced kidney injury, it supports the findings of Khan and co-workers who reported that obesity causes oxidative stress (Khan et al., 2006).

The derangements in plasma and urine concentrations of the renal function biomarkers, as recorded in this study, suggests that obesity is associated with both glomerular and renal tubular defects; a fact that was corroborated by the representative micrographic evidence showing atrophied glomerulus with tubular and interstitial vacuolation. Significant increase in the plasma level of renal function biomarkers is usually attributed to a compromise of the kidney's filtering capacity, hence the renal clearance of a substance is usually determined in order to assess glomerular filtration rate (Al-Qarawi et al., 2008; Valerie and Scanlon, 2007). This study demonstrated that the obesity-related reduction in renal clearance can be attributed to both glomerular filtration defects (micrographic evidence) and reduced renal perfusion (significantly lowered NO level and FEurea). The increased plasma concentrations of cystatin C, creatinine and urea can also be attributed to these deleterious factors. The consequence was a significantly reduced urinary excretion of these biomarkers. Furthermore, an important process of urea excretion is its secretion by the kidneys which is enhanced by urea transport proteins (Harlalka et al., 2007; Thangapandiyan et al., 2013). Therefore, the obesity-related reduction in the concentration of urine urea suggests a compromise of tubular secretory function resulting from a possible destructive alteration of the biochemical and or structural make up of tubular transport proteins. The representative micrographic evidence showed evidence of tubular vacuolation.

The findings of this study have been summarized with a figure that illustrates an apparent mechanism of oxidative stress in obesity-induced kidney injury (Fig. 5).

Attempts to improve scientific findings on the mechanism(s) of obesity-induced kidney injury to translational research should focus on the communication of the findings in a systematic fashion in order to enhance a synergy between basic science research and intervention research. This should consider acceptable conventions in obesity study in terms of terminology, scientific method as well as statistical

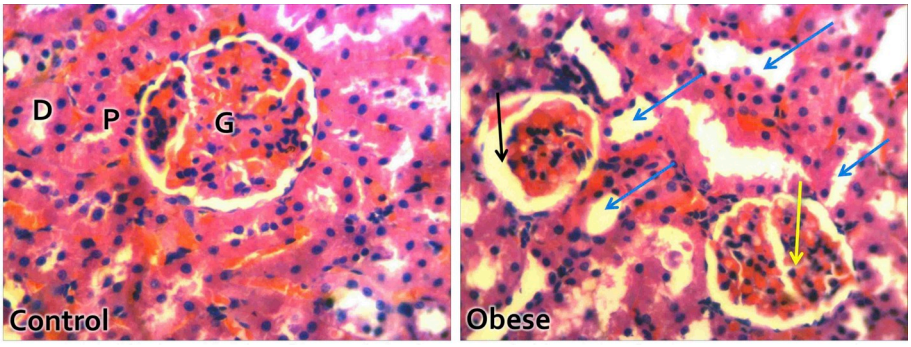

Fig. 4. Histological effects of obesity on the kidney of Wistar rats. **G** = glomerulus; **P** = proximal tubule; **D** = distal tubule; **Black arrow** = atrophied glomerulus; **Yellow arrow** = shrunken glomerulus; **Blue arrow** = vacuolated distal tubule and medullary interstitium (magnification = ×400).

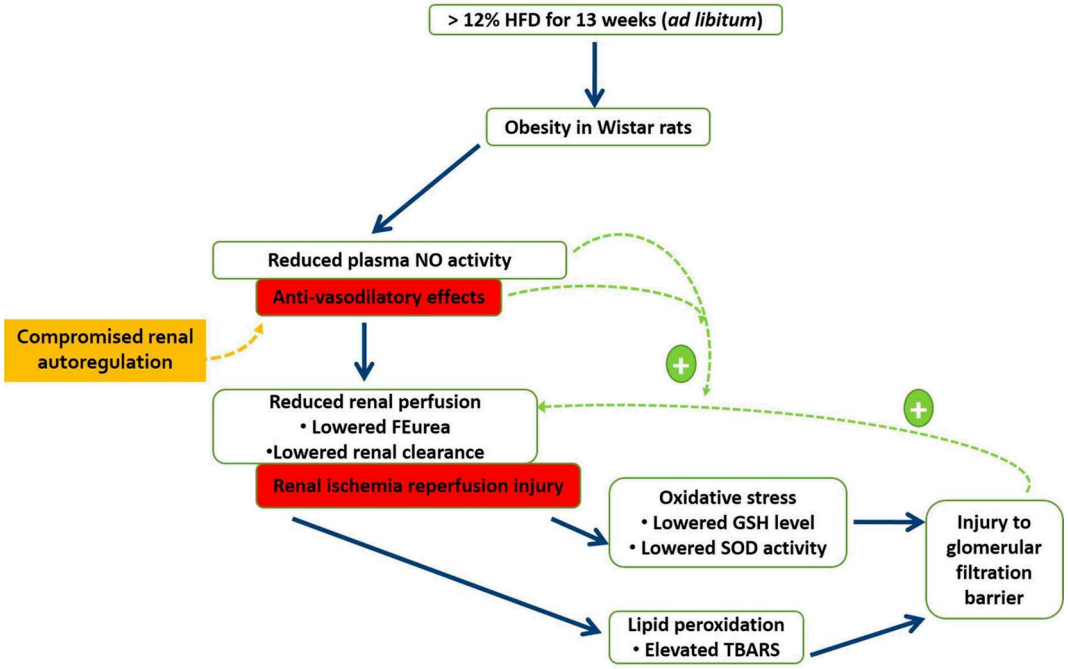

Fig. 5. Mechanism of oxidative stress in obesity-induced kidney injury. **NO** = nitric oxide; **FEurea** = fractional excretion of urea; **GSH** = reduced glutathione; **SOD** = superoxide dismutase; **TBARS** = thiobarbituric acid reacting substances; **Broken lines** (+) = positive feedback mechanisms.

approach. From the findings of this study, it is recommended that further novel studies on therapeutic strategies that will target ischemia reperfusion injury with a potential to sustain renal auto regulation mechanism and homeostatic level of circulating NO should be carried out. This may culminate in intervention strategies against obesity and or it comorbidities including renal dysfunction.

5. Conclusion

It was concluded that obesity induced renal injury with an associated oxidative stress through renal ischemia reperfusion injury in Wistar rats.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Glossary

BMI: body mass index
NO: nitric oxide
GSH: reduced glutathione
SOD: superoxide dismutase
TBARS: thiobarbituric acid reacting substances
FEurea: fractional excretion of urea
NFD: normal fat diet
HFD: high fat diet
G: glomerulus
P: proximal tubule
D: distal tubule
S.E.M: standard error of mean
i.m.: intramuscular
rpm: revolutions per minute
H & E: hematoxylin and eosin
NED: N-1-napthylethylenediamine dihydrochloride
EDTA: ethylene diamine tetra-acetic acid
TCA: trichloroacetic acid
TBA: thiobarbituric acid
ELISA: enzyme-linked immunosorbent assay
NIH: National Institutes of Health