Sildenafil, a phosphodiesterase-5-inhibitor decreased the oxidative stress induced by carbon tetrachloride in the rat kidney: A preliminary study

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ARTICLE INFO

ABSTRACT

Carbon tetrachloride (CCl_4) is a well-established nephrotoxin. Free radical generation has been described as one of the mechanisms of inducing its nephrotoxicity. This preliminary study investigated the protective effect of sildenafil (SILD) in CCl_4-induced oxidative stress in rat kidney. Thirty male albino wistar rats weighing between 150–200g were randomly divided into five groups, each consisting of 6 rats. Control group received physiological saline (10ml/kg, p.o.), another group received 0.5 ml/kg/i.p. of CCl_4 while three separate groups were pretreated with SILD at 5mg, 10mg and 20mg/kg respectively before CCl_4 challenge. Animals were sacrificed 24 hours after CCl_4 administration. Renal biomarkers were measured in the serum. In addition, antioxidant assays and histopathological studies were carried out. CCl_4 treatment produced no significant change in the serum levels of creatinine and BUN ($p>0.05$). However, CCl_4 significantly ($p<0.05$) reduced GSH level by 43.8% and increased lipid peroxidation by 37.5% ($p<0.05$) when compared to the group that received saline. Pretreatment with 10mg and 20mg/kg doses of SILD significantly raised GSH level by 28.0% and 35.7% respectively while lipid peroxidation was reduced by 50% ($p<0.05$) at 10mg/kg dose of SILD. Low dose of SILD (5mg/kg) was not effective as it caused a significant reduction of about 33.3% in GSH level ($p<0.05$). CCl_4 significantly lowered the activities SOD, CAT and GST by 63.5%, 60.0% and 47.5% respectively when compared with the control group while GPx activity was decreased slightly by 17.0% ($p>0.05$). SILD treatment at 10mg/kg significantly elevated the activities of SOD, CAT, GPx, and GST (by 30.9%, 56.0%, 26.9% and 76.5% respectively) as compared with the CCl_4-administered group while SILD at 5mg/kg led to reductions of 71.8%, 18.0%, 5.6% in the enzyme activities of SOD, CAT and GPx respectively. Sildenafil at highest dose of 20mg/kg significantly produced a further increase in the activities of SOD and CAT by 33.0% and 64.0% respectively. Data generated from this preliminary study suggested that high dose of sildenafil may protect against the oxidative stress induced by CCl_4 in the kidney of rats.

INTRODUCTION

The kidney is an organ involved in waste filtering and disposal in both human and animal bodies. It is also the target of so many xenobiotics during their metabolism. Carbon tetrachloride (CCl_4) is grouped under the class of the haloalkanes. It is commonly used as fumigants, anthelmintics and an intermediate in chlorofluorocarbons synthesis (McGregor and Lang, 1996). CCl_4 has been documented as a potent nephrotoxin found in animals (Ogeturk *et al.*, 2005). Its toxicity was found to result from its conversion by CYP2E1, a cytochrome P450, to the reactive trichloromethyl ($^*CCl_3$) and peroxy trichloromethyl ($^*OOCCl_3$) radicals (Al-Sayed *et al.*, 2015). These resultant radicals, then bind to the lipids of cell membrane to generate alkoxy (R^*) and peroxy radicals (ROO^*), which are highly toxic resulting into lipid peroxidation of cell membranes, cellular enzymes leakage

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(Khan *et al.*, 2010), oxidative damage to the DNA, and ultimately death of kidney cells (Hismiogullari *et al.*, 2015). Thus, processes involving reactive oxygen species (ROS) could be conveniently said to be contributing significantly to the etiology of CCl_4-induced damage in the kidney.

Sildenafil (SILD) is phosphodiesterase-5 (PDE5) selective inhibitor, which participates in the degradation of cyclic guanosine monophosphate (cGMP) and relaxation of the smooth muscle cells of the arterioles (Gibson, 2001). Our recent study provided experimental evidence on the ability of SILD to offer protection against adriamycin-induced hepatotoxicity via antioxidant mechanism (Adeyanju *et al.*, 2016). In addition, positive effect in animals preconditioned with SILD in auto-transplanted kidneys has been reported (Lledo-Garcia *et al.*, 2007). Moreover, SILD has also been found to reduce oxidative stress (Ebrahimi *et al.*, 2009) and exert anti-inflammatory effect (Rodriguez-Iturbe *et al.*, 2005) through NO/cGMP pathway. Its ameliorative effect on cisplatin-induced nephrotoxicity has also been demonstrated (Elberry *et al.*, 2014). In addition, Ali *et al.* (2011) has demonstrated the non-nephrotoxic potential of SILD. The study revealed that rats treated with sildenafil at a dose of 10mg/kg, via subcutaneous route for five days had normal renal architecture. In this preliminary study, an investigation was carried out to determine the possible mechanisms of protection of sildenafil against nephrotoxicity induced by CCl_4.

MATERIALS AND METHODS

Chemicals and reagents

Sildenafil citrate was purchased from Zurius Life sciences Pvt. Ltd. (India). Reduced glutathione (GSH), Ellman's reagent (5'5'-dithiobis-2-nitro benzoic acid), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were sourced from Sigma (St. Louis, MO, USA). Diagnostic assay kits for urea and creatinine determination were obtained from Randox Laboratories (Crumlin, U.K.). All other chemicals and reagents used in this experiment were of analytical grade.

Animals

Thirty male albino Wistar rats weighing 150-200g were obtained from the Department of Biochemistry, College of Medicine University of Ibadan, Ibadan, Nigeria. The acclimatization of the rats was for two weeks and was randomly divided into 5 groups (six rats per group). The rats were placed in a conventional room with a 12 hours light/12 hours dark cycle. The rats were housed in plastic cages, had access to commercial diet and water was given *ad libitum*.

Animal ethics

The animals used received humane care in compliance with standard guidelines set up for the Care and Use of Laboratory Animals (NRC, 2011) for animal experiments. The ethic regulations have been duly observed in line with the established national and institutional guidelines for the protection of animals' welfare during experiments. Ethical approval was obtained from the Ethical Committee of the College of Health Sciences, Osun State University, Osogbo, Nigeria and given ethical approval number UNIOSUN/HREC/2017/A/009.

Experimental design

Rats were divided into 5 groups of 6 animals. Group 1 (control) was given normal saline (10mL/kg, p.o.). Group II was administered with a single dose of CCl_4 (0.5mL/kg) to induce nephrotoxicity. Groups III, IV and V were pre-treated with SILD (5, 10 and 20 mg/kg) for 7 days. Rats were sacrificed 24 hours after the CCl_4 challenge. Section of the kidney was excised, fixed in 10% formalin for histopathology examination.

Preparation of tissue homogenates

Rats were sacrificed by cervical dislocation. Blood samples were collected into plain bottles prior to animal sacrifice and centrifuged at 3000g at room temperature for 3 minutes to separate serum. The kidney was rapidly dissected and removed, rinsed in 1.15% KCl, blotted with Whatman filter paper and weighed. It was later placed in an ice cold phosphate buffer pH 7.4 and homogenized in a Teflon-glass homogenizer. The centrifugation of the kidney homogenate was done at 12,000 *g* for 15 minutes at 4°C The post mitochondrial fractions obtained after centrifugation were stored at 4°C and used later for biochemical assays.

Measurement of serum biomarkers for kidney function

The creatinine and blood urea nitrogen (BUN) serum levels were determined using commercially available kits.

Estimation of antioxidant parameters

Assay of glutathione peroxidase (GPx) activity

The GPx activity was determined following the method of Lawerence and Burk (1961). 100 μL of kidney homogenate was mixed with 800μL of 100 mM potassium phosphate buffer (pH 7.4), which contains the mixture of 1 mM NaN_3, 1mM EDTA, 0.2 mM NADPH, 1U/mL GSH reductase and 1 mM GSH. The reaction was started after 5mins by adding 2.5 mM H_2O_2 (100 μL) to the above mixtures. The absorbance changes was taken within 3 minutes and recorded at 340 nm. The enzyme activity was calculated and the result was expressed by nmol NADPH/minute/mg protein.

Assay of glutathione S-transferase (GST) activity

The activity of GST was measured by the method of Habig *et al.* (1974). Briefly, 100 μL of the kidney homogenate was mixed thoroughly with 880 μL of phosphate buffer (pH 6.5, 100 mM potassium) which contains the mixtures of 20 μL of 50 mM 1-chloro-2,4-dinitrobenzene and 100 mM GSH, and. Changes in absorbance was determined within 3 minutes at 340 nm. The calculated enzyme activity was expressed by nmol1-chloro-2,4-dinitrobenzene-GSHconjugated formed/minute/mg protein.

Assessment of lipid peroxidation

Lipid peroxidation assay was determined by the method of Ohkawa *et al.* (1979). The formation of thiobarbituric reactive substances (TBARS) formed was measured at 532 nm. The malonylaldehyde (MDA) level was calculated from the absorbance according to the method of Adam-Vizi and Seregi (1982) and expressed as nmol MDA/mg protein.

Determination of reduced glutathione (GSH)

The total sulfhydryl groups, protein-bound sulfhydryl groups, and free sulfhydryl groups (such as reduced GSH) in biological samples can be determined using Ellman's reagent (DTNB) as described by Jollow *et al.* (1974). A 2.5mL of aliquot was mixed with

an equal volume of 4% sulfosalicylic acid to facilitate deproteinization. The mixture was centrifuged at $14,000 \times g$ for 15 min at 4°C. Then, 0.5 mL of the supernatant was mixed with 4.5 mL of Ellman's reagent and absorbance of the samples taken at 412 nm. The blank was prepared by mixing 0.5 mL of diluted precipitating reagent (diluted twice with 0.1M phosphate buffer, pH 7.4) with 4.5 mL of Ellman's reagent.

Determination of superoxide dismutase (SOD) activity

The level of SOD activity was determined spectrophotometrically as described by Misra and Fridovich (1972). One unit of SOD activity is defined as the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adrenochrome in 1 min. The activity was expressed as units/mg protein.

Determination of catalase (CAT) activity of samples

The catalase activity was estimated according to the method of Asru (1972). The H_2O_2 contents of the withdrawn solutions were subsequently determined kinetically at 25°C. The results expressed as mmol H_2O_2 consumed/min/mgprotein.

Determination of protein content of samples

Protein determination in tissues was carried out using (Lowry *et al.*, 1951) and the standard used was bovine serum albumin.

Histological assessment

A section of rat's kidney from different rats groups were fixed in 10% neutral formalin solution. They were dehydrated in graded alcohol and embedded in paraffin. Fine sections obtained were mounted on glass slides and counter-stained with hematoxylin-eosin for light microscopic analyses. The slides were thereafter examined by a histopathologist who was blinded to the treatment groups after which photographs were taken.

Data analysis

Results were expressed as mean ± standard error of mean. The statistical analysis were evaluated using one-way analysis of variance of Statistical Package for Social Sciences software for Windows version 16 (SPSS Inc., Redmond, WA, USA). Post hoc testing was done for intergroup comparisons using the least significant difference. The level of statistical significance was $p<0.05$.

RESULTS

BUN and creatinine

Effect of administration of SILD and CCl_4 on serum creatinine and BUN levels is shown in Table 1. Notably, administration of CCl_4 alone and the pretreatment with various doses of SILD produced no significant alterations on the levels of BUN and serum creatinine as compared to the normal and the group that received CCl_4 respectively.

Table 1: Effect of SILD on CCl_4-induced changes in the activities of the marker enzymes of kidney function.

Treatment group	Urea	Creatinine
Control (NaCl)	17.1 ± 0.3	1.2 ± 0.03
CCl_4 (0.5 mL/kg)	$16.3 \pm 0.3 \ (4.9)^a$	$1.2 \pm 0.15 \ (0)^a$
SILD (5 mg/kg) + CCl_4 (0.5 mL/kg)	$16.0 \pm 0.43 \ (1.9)^b$	$1.1 \pm 0.15 \ (9.0)^b$
SILD (10 mg/kg) + CCl_4 (0.5 mL/kg)	$13.8 \pm 0.99^{**} \ (18.1)^b$	$1.2 \pm 0.12 \ (0)^b$
SILD (20 mg/kg) + CCl_4 (0.5 mL/kg)	$16.0 \pm 2.7 \ (1.9)^b$	$0.7 \pm 0.1^{**} \ (71.0)^b$

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. *Significantly different from control ($p<0.05$); **significantly different from CCl_4-treated rats ($p<0.05$). Values in parenthesis represent % change; [a]% change relative to control; [b]% change relative to CCl_4.

Effect of treatment on GSH and lipid peroxidation

A significant reduction of 43.8% was observed in the level of GSH in CCl_4-administered rats when compared to the control group. Administration of low dose of 5mg/kg of SILD caused a reduction of about 33.3% in the level of GSH ($p<0.05$). However, higher doses of 10mg and 20mg/kg of SILD significantly raised GSH level by 28.0% and 35.7% respectively. CCl_4 treatment increased the MDA level (an index of lipid peroxidation) by 37.5% which was statistically different from the control group. Pretreatment with SILD at 10mg/kg reduced the CCl_4-induced elevation in renal MDA by 50% ($p<0.05$) when compared with the CCl_4-intoxicated rats (Table 2).

Table 2: Effect of SILD on CCl_4-induced changes in GSH and MDA.

Treatment group	GSH (µg/gtissue)	MDA (nmol/mgprotein)
Control (NaCl)	32.0 ± 2.5	2.5 ± 0.14
CCl_4 (0.5mL/kg)	$18.0 \pm 4.4^* \ (43.8)^a$	$4 \pm 1.7^* \ (37.5)^a$
SILD (5mg/kg) + CCl_4 (0.5mL/kg)	$12.0 \pm 6.2^{**} \ (33.3)^b$	$2.6 \pm 0.9 \ (35.0)^b$
SILD (10mg/kg) + CCl_4 (0.5mL/kg)	$25.0 \pm 3.5^{**} \ (28.0)^b$	$2.0 \pm 0.1^{**} \ (50.0)^b$
SILD (20mg/kg) + CCl_4 (0.5mL/kg)	$28.0 \pm 2.5^{**} \ (35.7)^b$	$2.7 \pm 0.4^{**} \ (32.5)^b$

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. *Significantly different from control ($p<0.05$); **Significantly different from CCl_4-treated rats ($p<0.05$). Values in parenthesis represent % change; [a]% change relative to control; [b]% change relative to CCl_4.

Effect of treatment on antioxidant enzymes

CCl_4 challenge produced a significant decrease in the activities of SOD, CAT and GST by 63.5%, 60.0% and 47.5% respectively when compared with the group that received physiological saline while GPx activity was decreased slightly by 17.0% (p>0.05) following the administration of CCl_4. SILD treatment at 10mg/kg resulted in a significant elevation in CAT, GPx, and GST activities (by 56.0%, 26.9% and 46.8% respectively) and non-significant increase in SOD activity (30.9%) as compared with the CCl_4-treated group. The highest dose of SILD (20mg/kg) increased significantly the activities of catalase and GST by 64.0% and 66.7% respectively while SOD and GPx activities were non-significantly increased by 33.0% and 11.8% respectively (Table 3).

Histopathological examination

The photomicrographs of the histology done on the kidney tissue of rats in the various treatment groups are presented in Figure 1. The toxicity of CCl_4 in the kidney was characterized by severe diffuse tubular and glomerular degeneration. CCl_4-intoxicated rats treated with various doses of SILD showed no visible lesions.

Table 3: Effect SILDon CCl_4-induced alterations in antioxidant parameters.

Treatment group	SOD (units/gtissue)	CAT H_2O_2 (μmole consumed/min)	GPx (nmol/mgprotein/min)	GST (units/gtissue)
Control (NaCl)	10.4 ± 1.2	10 ± 0.18	23.0 ± 5.8	8.0 ± 0.76
CCl_4 (0.5mL/kg)	$3.8 \pm 2.3^*$ (63.5)[a]	$0.4 \pm 0.01^*$ (60.0)[a]	19.0 ± 3.9 (17.0)[a]	$4.2 \pm 1.4^*$ (47.5)[a]
SILD (5mg/kg) + CCl_4 (0.5mL/kg)	$1.07 \pm 1.1^{**}$ (71.8)[b]	$0.33 \pm 0.01^{**}$ (18.0)[b]	$18.0 \pm 8.$ (5.6)[b]	13.7 ± 3.7 (69.34)[b]
SILD (10mg/kg) + CCl_4 (0.5mL/kg)	5.5 ± 2.4 (30.9)[b]	$0.9 \pm 0.18^{**}$ (56.0)[b]	$26.0 \pm 3.9^{**}$ (26.9)[b]	$17.9 \pm 0.6^{**}$ (76.5)[b]
SILD (20mg/kg) + CCl_4 (0.5mL/kg)	5.7 ± 2.9 (33.0)[b]	$1.1 \pm 0.46^{**}$ (64.0)[b]	17.0 ± 5.0 (11.8)[b]	$12 \pm 2.3^{**}$ (66.7)[b]

Values expressed as mean ± standard error of mean (SEM) for five rats in each group. *Significantly different from control (p<0.05); **Significantly different from CCl_4-treated rats (p<0.05). Values in parenthesis represent % change; [a]% change relative to control; [b]% change relative to CCl_4.
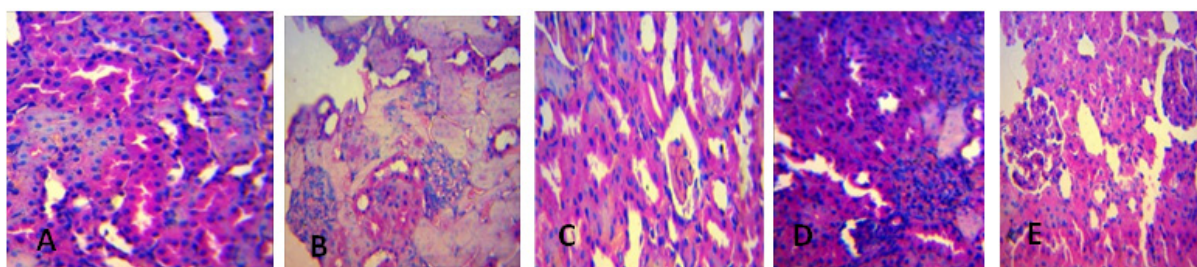

Fig. 1: Kidney section (x 400) of rat treated with **[A]** normal saline (10mL/kg) without visible lesions; **[B]** CCl_4 (0.5mL/kg) showing a severe diffuse tubular and glomerular degeneration; **[C]** CCl_4 + SILD (5mg/kg) showing no visible lesions; **[D]** CCl_4 + SILD (10mg/kg) showing no visible lesions; **[E]** CCl_4 + SILD (20mg/kg) showing no visible lesions.

DISCUSSION

In this preliminary study, we did not observe any effect of CCl_4 on kidney function as the levels of BUN and serum creatinine were not increased after CCl_4 challenge. Our submission here is that, the short-term exposure of rats to CCl_4 might be a contributing factor to this observation. In addition, the estimation of the renal function which was done just 24 hours after the administration of CCl_4 may also not be sufficient to present a significant increase in creatinine and urea serum levels. Urea and creatinine are not reliable markers of early renal injury as they can remain unchanged at the early stage of the disease. Since oxidative stress parameters were significantly affected and histology revealed morphological damage, it implies there is an ongoing injury involving oxidative stress mechanism. Urea and creatinine levels usually become significantly elevated when renal damage is advanced so histology and oxidative stress parameters are sufficient to imply nephrotoxicity or renal damage with or without elevation of urea and creatinine. A study by Tirkey *et al.* (2005) and Zimmerman *et al.* (1983) also showed similar observation regarding the above observation on urea and creatinine levels in our present study. Ogawa *et al.* (1992) reported that CCl_4 challenge did not cause an increase in BUN level in Balb/c mice until after 12 weeks of its administration. Although our data showed no alterations in the renal function after 24 hours of CCl_4 administration, the short-term exposure still resulted into a significant oxidative stress in the kidney. CCl_4 has been reported to cause oxidative stress in the kidney with evidence of increase in lipid peroxidation and altered antioxidant status (Manna *et al.*, 2006). Pre-treatment with SILD for 7 days obviously reduced the extent of lipid peroxidation induced by CCl_4. This ability of SILD to ameliorate the level of lipid peroxidation induced by CCl_4 treatment is in agreement with the report of Cadirci *et al.* (2011) who demonstrated that SILD decreased MDA level in the kidney of cecal ligation and puncture-induced septic rats. In addition, SILD was found to decrease the levels of MDA in the kidney of rats subjected to ischemia-reperfusion injury (Küçük *et al.*, 2012) and studies by Morsy *et al.* (2014) also documented the efficacy of SILD as it significantly decreased the elevated MDA level in gentamicin-toxicity. Our finding in the present study is also in harmony with the reports of many researchers who had also shown

that treatment with CCl_4 significantly reduced renal GSH and this could alter the redox status of the cell. The reaction of the resultant metabolites from CCl_4 with sulfhydryl groups of GSH and protein thiols has been attributed to this (Khan *et al.*, 2009). The enhanced lipid peroxidation observed in this study might also be responsible for the reduced GSH level. However, administration of SILD for 7 days prior to CCl_4 treatment significantly raised this reduced GSH level especially at the higher doses used in this study.

Furthermore, renal SOD, catalase, GPx and GST activities respectively were significantly decreased in the CCl_4-treated rats. The reduction in the activity of SOD could be as a result of the increased lipid peroxidation observed earlier or the antioxidant enzymes inactivation which would ultimately result in superoxide radicals' accumulation, further promoting lipid peroxidation. In contrast, treatment with sildenafil demonstrated ability to increase SOD and CAT activities as also documented by Abdel-Latif *et al.* (2013) and Beheshtian *et al.* (2008) respectively. The higher doses, 10mg and 20mg/kg of SILD used in our study consistently protected the antioxidant enzymes of the kidney from CCl_4-induced oxidative stress. From histology point of view, pretreatment with SILD offered remarkable protection against kidney damage as shown in the intact renal architecture with no visible lesions.

In conclusion, data from this preliminary study supports the hypothesis that oxidative stress plays an important role in the mechanism of CCl_4-induced nephrotoxicity and that SILD has therapeutic potential in preventing renal injury induced by CCl_4 possibly via antioxidant mechanism.

FINANCIAL SUPPORT AND SPONSORSHIP

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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