Clofibrate, a Peroxisome Proliferator–Activated Receptor-Alpha (PPARα) Agonist, and Its Molecular Mechanisms of Action against Sodium Fluoride–Induced Toxicity

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Abstract

Sodium fluoride (NaF) is one of the neglected environmental pollutants. It is ubiquitously found in the soil, water, and environment. Interestingly, fluoride has been extensively utilized for prevention of dental caries and tartar formation, and may be added to mouthwash, mouth rinse, and toothpastes. This study is aimed at mitigating fluoride-induced hypertension and nephrotoxicity with clofibrate, a peroxisome proliferator–activated receptor-alpha (PPARα) agonist. For this study, forty male Wistar rats were used and randomly grouped into ten rats per group, control, sodium fluoride (NaF; 300 ppm) only, NaF plus clofibrate (250 mg/kg) and NaF plus lisinopril (10 mg/kg), respectively, for 7 days. The administration of NaF was by drinking water *ad libitum*, while clofibrate and lisinopril were administered by oral gavage. Administration of NaF induced hypertension, and was accompanied with exaggerated oxidative stress; depletion of antioxidant defence system; reduced nitric oxide production; increased systolic, diastolic and mean arterial pressure; activation of angiotensin-converting enzyme activity and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); and testicular apoptosis. Treatment of rats with clofibrate reduced oxidative stress, improved antioxidant status, lowered high blood pressure through the inhibition of angiotensin-converting enzyme activity, mineralocorticoid receptor over-activation, and abrogated testicular apoptosis. Taken together, clofibrate could offer exceptional therapeutic benefit in mitigating toxicity associated with sodium fluoride.

Keywords: Sodium fluoride toxicity; Hypertension; Oxidative stress; Cell signalling; Apoptosis; Antihypertensive

Introduction

Sodium fluoride (NaF) is one of the neglected environmental pollutants in the world. It is ubiquitously found in the soil, water and the environment [65]. Fluoride is present in mouthwash, mouth rinse and toothpastes and has been used medically for the prevention of dental caries and tartar formation [39, 55]. Human activities have been found to enhance the release of fluorides from the environment into groundwater [50, 57, 95]. Previously in our laboratory, we have shown that free radical generation, reactive oxygen species (ROS) and oxidative stress are the major culprits in fluoride-induced nephrotoxicity [64]. Lipid peroxidation products and ROS are also known to play key roles in the pathophysiology of fluoride toxicities leading to damage of DNA, lipids and proteins and enhance induction of apoptosis [14, 73, 82]. Fluoride toxicity has also been shown to precipitate skeletal fluorosis leading to osteoporosis of the bones and osteosclerosis [37, 80]. Other findings have documented target organs and tissues affected by NaF toxicity to include the kidney, brain, liver, testes and blood [1, 20, 69, 71]. Interestingly, toxicity associated with NaF has been shown to precipitate hypertension and cardiovascular complications from our previous studies [64,65,66,67].

Hypertension is one of the most silent killers in the world. It has a plethora of risk factors including dietary, genetic and environmental [9, 72]. Globally, billions of dollars are spent annually for the management and treatment of hypertension in low-income and middle-income countries (LMICs) of the world [28]. For the past two decades, the impact of financial burden coupled with side effects from the antihypertensive is alarming [44, 58]. However,

nutraceuticals and phytochemicals have been used in ameliorating fluoride toxicity [65,66,67]. Recently, drug repurposing is increasingly becoming attractive for the management of various disease conditions [12, 75]. In the present study, we hypothesized that clofibrate, a PPARα agonist, could be repurposed for mitigating hypertension and complications arising from sodium fluoride toxicity.

The lipid sensor peroxisome proliferator–activated receptor-alpha (PPAR- α) is a well-known master regulator of lipid metabolism [60]. The fibrates including clofibrate are a class of drugs that have been established to lower high blood cholesterol and prevent cardiovascular diseases [48, 54, 88]. Interestingly, previous studies have reported the antihypertensive effect of clofibrate on spontaneously hypertensive rats (SHR), salt-loaded hypertensive rats and saline-induced endothelial dysfunction [17, 78, 97]. The renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood pressure and body fluid, which contribute to the pathophysiology of hypertension and cardiovascular/renal diseases [5, 6, 61]. In the present study, we examined the molecular mechanism of action of clofibrate as a novel antihypertensive agent against NaF-induced hypertension in an experimental rat model.

We hypothesized that mineralocorticoid receptor (MCR)/angiotensin-converting enzyme (ACE)/kidney injury molecule (Kim-1)/angiotensin II receptor type 1 (AT1 receptor) signalling pathway could serve as molecular therapeutic targets for clofibrate in reducing high blood pressure and mitigating renal damage associated with fluoride toxicity.

Materials and Methods

Chemicals

Clofibrate, trichloroacetic acid (TCA), sodium hydroxide, O-dianisidine, hydrogen peroxide (H_2O_2), xylenol orange (XO), potassium hydroxide, reduced glutathione (GSH), oxidized glutathione (GSSG), sodium fluoride, thiobarbituric acid (TBA) and 1,2-dichloro-4-nitrobenzene were purchased from Sigma (St. Louis, MO, USA). Normal goat serum, biotinylated antibody and horseradish peroxidase (HRP) system were purchased from (KPL, Inc., Gaithersburg, MD, USA). Anti-nuclear factor kappa beta (NF-κB), mineralocorticoid receptor (MCR), angiotensin-converting enzyme (ACE), angiotensin II receptor type 1 (AT1 receptor) and caspase 3 were purchased from Sigma-Aldrich Bryanston, Sandton, 2021, South Africa. 3,3′-Diaminobenzidine (DAB) tablets were purchased from AMRESCO LLC. (OH, USA). All other chemicals were of analytical grade.

Experimental Animals and Design

For this study, forty male Wistar rats (100–110 g) were used and randomly grouped into ten rats per group, control (normotensive), NaF (300 ppm; hypertensive) only, hypertensive plus clofibrate (250 mg/kg) and hypertensive plus lisinopril (10 mg/kg), respectively, for 7 days. The dosage of NaF was chosen according to our previous study [64], clofibrate [97] and lisinopril [59]. The administration of NaF was by drinking water ad libitum, while clofibrate and lisinopril were administered by oral gavage. The rats were kept in wire mesh cages under controlled light cycle (12-h light/12-h dark) and liberally supplied with commercial rat chow and water ad libitum. The rat chow was purchased from Ladokun Feeds Limited, Ibadan, Nigeria. The final weights of the rats were taken 24 h (day 7) before the sacrifice which was on day 8.

Blood Pressure Measurement

The blood pressure parameters of the rats were taken on day 8 with an automated blood pressure monitor (CODA S1, Kent Scientific Corporation, CT, USA). The systolic (SBP), diastolic (DBP) and mean arterial (MAP) blood pressures were determined non-invasively in conscious animals by tail plethysmography as recently reported from our laboratory [68].

Blood Sample Collection and Serum Preparation

Rats were humanely handled, and about 3 ml of blood was collected through the retro-orbital venous puncture with the aid of capillary tubes into plain bottles and allowed to clot. The clotted blood was centrifuged at 4000 × *g* for 10 min. Clear serum was harvested and stored at 4°C until analysis. Ethical regulations were followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments [27]. The kidney, testes and epididymis were harvested and weighed for the determination of organ weight and relative organ weights, respectively.

Determination of Serum Testosterone

Serum testosterone was assayed using an enzyme immunoassay kit (DRG Diagnostics, Germany). The sensitivity of the testosterone assay was 0.08% taking into consideration other androgen derivatives like methyl testosterone, androstenedione and 5 alpha-dihydrotestosterone. We also adhered strictly to variation in intra-assay coefficient following manufacturer's instruction.

Preparation of Renal and Testicular Post-mitochondrial Fractions

For the preparation of tissue homogenates, the kidney and testes were quickly excised, rinsed, blotted with filter paper, weighed, chopped into bits and homogenized with homogenizing buffer (0.1-M phosphate buffer, pH 7.4) using a Teflon homogenizer for thirty strokes each. The resulting homogenate was centrifuged at 10,000 × *g* for 10 min with a cold centrifuge at −4°C to obtain post-mitochondrial fractions (PMFs). The supernatants (PMFs) were used for biochemical assays.

Biochemical Assays

Determination of Antioxidant Defence System

The superoxide dismutase (SOD) assay was carried out by the method of Misra and Fridovich [59], with slight modification from our laboratory [68]. The glutathione peroxidase (GPx) activity was also measured according to Beutler et al. [8], while the activity of glutathione S-transferase (GST) was estimated by the method of Habig et al. [34] using 1-chloro-2,4-dinitrobenzene as substrate. Further, the reduced glutathione (GSH) content was estimated by the method of Jollow et al. [47], while the protein thiol (PSH) and non-protein thiol (NPSH) contents were determined as previously described by Ellman [22].

Estimation Makers of Oxidative Stress

The hydrogen peroxide (H_2O_2) generation was estimated according to the method of Wolff [90]. The vitamin C contents were measured as earlier described by Jacques-Silva et al. [43].

Protein carbonyl (PCO) contents as index of protein oxidation in the renal and testicular tissues were measured using the method of [76]. The malondialdehyde (MDA) content as an index of lipid peroxidation was quantified in the PMFs of testicular and renal tissue according to the method of Varshney and Kale [86]. The absorbance was measured against blank at 532 nm. Lipid peroxidation index was calculated with a molar extinction coefficient of 1.56×10^5/M/cm. The advanced oxidation protein product (AOPP) contents were determined, and contents of AOPP were calculated using the extinction coefficient of 261 cm^{-1} mM^{-1} and expressed as μmoles/mg protein as described by Kayali et al. [51]. The serum nitric oxide concentrations were measured spectrophotometrically at 548 nm according to the method of Olaleye et al. [62].

Protein Determination

Serum and tissue protein contents were assayed according to Biuret's method as described by Gornal et al. [30].

Histopathology

Kidney and testicular tissues were fixed in 10% formalin and Bouin's solution**,** respectively, embedded in paraffin wax, and sections of 5–6 mm in thickness were made and thereafter stained with haematoxylin and eosin (H&E) as previously described [21].

Immunohistochemistry

The immunolocalization of mineralocorticoid receptor (MCR), nuclear factor kappa beta (NF-κB), angiotensin-converting enzyme (ACE), angiotensin II receptor type 1 (AT1 receptor) and anti-caspase 3 was determined as earlier reported by Oyagbemi et al. [68] in the kidney and testes, respectively. Kidney and testes samples were fixed with 4% formaldehyde, embedded in paraffin and sectioned at a thickness of 5 μm. Antigen retrieval was carried out in 10-mM citrate buffer (pH 6.0) for 25 min, followed by subsequent peroxidase quenching in 30% H_2O_2/methanol. The sections were blocked in 2% milk for 1 h to enhance specific binding of antigen-antibody and probed with the following antibodies overnight at room temperature, NF-κB (1:500, Sigma, South Africa), MCR (1:500, Sigma, USA), AT1 receptor (1:500, Sigma, South Africa), ACE (1:500, Sigma, South Africa) and anti-caspase 3 (1:500, Sigma, South Africa), for the kidney and testes, respectively, using polyclonal antibodies for 16 h at 4 °C. After washing, the sections were incubated for 2 h at room temperature in the appropriate biotinylated secondary antibodies. The immune-positive reactions were enhanced with 3,3′-diaminobenzidine (DAB, AMRESCO LLC., OH, USA). Sections were observed with a light microscope (Leica LAS-EZ®) using Leica software application suite version 3.4 equipped with a digital camera. Immunoreactivity was quantified using ImageJ which measures immune-positive regions relative to total tissue areas.

Statistical Analysis

Our data were analysed with one-way ANOVA followed by Tukey's test to compare each group mean with one another. All values are expressed as mean ± SD. The test of significance between two groups was estimated by Student's *t* test. The level of significance of $p<0.05$ was taken as statistically significant.

Results

Body Weight and Relative Organ Weight

There were noticeable variations in the final body weight of rats across the experimental groups, although, not significant. It was observed that the body weight of sodium fluoride–untreated rats was higher than the control and those treated with clofibrate or lisinopril (Table 1). The epididymal weight and the relative epididymal weight of sodium fluoride alone group increased ($p<0.05$) significantly when compared to the control. However, there was no significant increase in both the kidney weight and relative kidney weight of toxicant group compared to the control rats (Table 1). Surprisingly, there were significant ($p<0.05$) decreases in the relative epididymal and relative testicular weights of NaF-treated rats with lisinopril **(Table 1).**

Table 1 Effect of clofibrate on body weight, organs weight and relative body weight of hypertensive rats

	Control	NaF	NaF + clofibrate	NaF + lisinopril
Final body weight	124.30 ± 17.10	151.348 ± 10.23	143.8 ± 15.48	122 ± 24.60
Testes weight (g)	1.72 ± 0.45	2.42 ± 0.20[a]	2.05± 0.15	1.60 ±0.45[b]
Epididymis weight (g)	0.29 ±0.18	0.41 ± 0.06[a]	0.23 ± 0.05	0.28 ±0.17[b]
Kidney weight (g)	1.06 ± 0.15	1.13 ± 0.13	1.26 ± 0.22	0.93 ± 0.13
Relative epididymis weight (g)	0.22 ± 0.12	0.27 ± 0.05[a]	0.16 ±0.02[b]	0.13 ± 0.09[b]
Relative kidney weight (g)	0.83 ± 0.15	0.73 ± 0.13	0.8 ± 0.06	0.77 ± 0.05
Relative testes weight (g)	1.53 ± 0.41	1.61 ±0.22	1.43 ±0.15	1.30 ±0.18[b]

Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while superscript (b) indicates significant difference when compared to clofibrate alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups in each row. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD ($n=10$)

Renal Markers of Oxidative Stress and Reactive Oxygen Species Generation

The results in Table 2 showed significant ($p<0.05$) increases in H_2O_2 generation, MDA and PCO contents of NaF-treated rats in comparison to rats co-treated with clofibrate or lisinopril. Further, there were concomitant significant ($p<0.05$) reductions in the values of H_2O_2 generation, MDA and PCO of rats administered clofibrate or lisinopril, which was indicative of free radical scavenging activity of clofibrate (Table 2). Again, the values of protein thiol (PSH) of rats treated with lisinopril reduced ($p<0.05$) significantly relative to the control and NaF group (Table 2). On the other hand, the values obtained for NPSH of NaF group decreased ($p<0.05$) significantly when compared to the control rats. However, there was no statistically significant ($p>0.05$) difference in the values of renal NPSH of NaF group treated with clofibrate or lisinopril (Table 2).

Table 2. Effect of clofibrate on renal markers of oxidative stress in sodium fluoride–induced hypertension

Kidney	Control	NaF	NaF + clofibrate	NaF + lisinopril
H_2O_2	94.19 ± 9.74	115.07 ± 7.52[a]	87.12 ± 9.51[b]	85.49 ± 8.33[b]
MDA	1.70 ± 0.65	2.30 ± 0.19[a]	1.60 ±0.53	1.90 ± 0.75
PCO	6.49 ± 0.56	9.97 ±1.48[a]	5.21 ± 1.06[a]	9.05 ± 0.25
PSH	76.34 ±8.11	87.04 ± 13.18	79.89 ± 8.85	48.60 ± 8.25[a,b]
NPSH	44.21 ± 6.93	36.98 ± 2.80[a]	42.89 ± 3.80	40.17 ± 1.05

Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to clofibrate alone at $p<0.05$ ($n=10$). Superscripts denote statistical difference across all groups in each row. Values are presented as mean ± SD ($n=10$). Abbreviations: H_2O_2 hydrogen peroxide generation (µmol/mg protein), *MDA* malondialdehyde (µmol of MDA formed/mg protein), *PCO* protein carbonyl (µmol/mg protein), *PSH* protein thiol (µmol/mg protein), *NPSH* non-protein thiol (µmol/mg protein)

Renal Antioxidant Defence System

The activity of SOD in rats treated with either clofibrate or lisinopril increased ($p<0.05$) significantly when compared with the control and NaF group (Table 3). Parallel to the observed activity of SOD, the renal activity of GPx and GST did not differ significantly in NaF group and treated rats in comparison to the control rats (Table 3). However, we observed a slight increase in the activity of GPx, though not statistically significant across all the experimental groups (Table 3). Contrary to the results of antioxidant enzyme activities, the contents of GSH decreased ($p<0.05$) significantly in NaF group, while significant improvement in renal GSH contents was obtained in rats treated with either clofibrate or lisinopril (Table 3). Furthermore, the renal vitamin C content of NaF group and rats treated with clofibrate increased ($p<0.05$) significantly in comparison to control (Table 3).

Table 3 Effect of clofibrate on levels of renal antioxidant enzymes in sodium fluoride–induced hypertension

Kidney	Control	NaF	NaF + clofibrate	NaF + lisinopril
SOD	41.82 ± 2.73	45.49 ± 7.55	86.88 ± 3. 63[a,b]	53.76 ± 6.00[a,b]
GP_X	207.05 ± 8.98	209.01 ± 4.64	208.61 ± 4.20	208.61 ± 4.60
GST	3.15 ± 0.96	2.27 ± 0.58	2.77 ± 0.59	2.67 ± 0.58
GSH	132.55 ± 12.61	77.44 ± 6.34[a]	125.62 ± 14.19[b]	119.32 ± 13.15[b]
VIT C	1.80 ± 0.09	1.99 ± 0.11[a]	2.05 ± 0.10[a]	1.91 ± 0.04

Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to clofibrate alone at $p<0.05$ ($n=10$). Superscripts denote statistical difference across all groups in each row. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD ($n=10$). Abbreviations: *SOD* superoxide dismutase (units/mg protein), *GPx* glutathione peroxidase (units/mg protein), *GST* glutathione-S-transferase (mmole 1-chloro-2,4-dinitrobenzene-GSH complex formed/min/mg protein), *GSH* reduced glutathione (µmol/mg protein), vitamin C (µmol/mg protein)

Testicular Antioxidant Defence System

In another experiment, the testicular SOD, GPx and GST activities were found to reduce ($p<0.05$) significantly in NaF-induced hypertensive rats (Table 4). However, there was

remarkable improvement in the testicular activities of GPx and GST of rats treated with clofibrate or lisinopril (Table 4). Also, there was noticeable reduction in the content of testicular GSH of sodium fluoride groups, although the reduction was not statistically different from the control or NaF-treated rats. Similarly, the testicular vitamin C contents decreased ($p<0.05$) significantly in NaF and rats administered clofibrate or lisinopril when compared with control rats (Table 4).

Table 4. Effect of clofibrate on testicular antioxidant enzymes in sodium fluoride–induced hypertension

Testes	Control	NaF	NaF + clofibrate	NaF + lisinopril
SOD	85.15 ± 13.94	45.15 ± 4.43[a]	46.40 ±5.54[a]	43.37 ± 5.52[a]
GP_X	211.01 ± 2.58	205.79 ± 2.88[a]	210.55 ± 2.75[b]	210.0 ± 2.67[b]
GST	2.37 ± 0.39	1.63 ± 0.63[a]	1.62 ± 0.19[a]	2.44 ± 0.58[b]
GSH	87.22 ± 8.55	83.03 ± 3.97	85.79 ± 6.20	89.77 ± 8.17
VIT C	4.67 ± 0.61	1.59 ± 0.14[a]	1.78 ± 0.16[a]	1.72 ± 0.15[a]

Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to clofibrate alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups in each row. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD ($n=10$). Abbreviations: *SOD* superoxide dismutase (units/mg protein), *GPx* glutathione peroxidase (units/mg protein), *GST* glutathione S-transferase (mmole 1-chloro-2,4-dinitrobenzene-GSH complex formed/min/mg protein), *GSH* reduced glutathione (μmol/mg protein), vitamin C (μmol/mg protein)

Testicular Markers of Oxidative Stress

The testicular H_2O_2 generation and PCO content increased ($p<0.05$) significantly in NaF-intoxicated rats with concomitant reduction in the H_2O_2 generation and PCO content of rats that were treated with clofibrate or lisinopril (Table 5). The values of MDA content as index of lipid peroxidation product also reduced ($p<0.05$) significantly in rats treated with clofibrate. However, the observed increase in the MDA content of NaF-intoxicated rats was not statistically significant (Table 5). However, we observed that decrease in the values of testicular NPSH and PSH of NaF alone and NaF-treated rats did not show significant difference (Table 5).

Table 5. Effect of clofibrate on testicular markers of oxidative stress in sodium fluoride–induced hypertension

Testes	Control	NaF	NaF + clofibrate	NaF + lisinopril
H_2O_2	55.16 ± 9.38	66.65 ± 3.68[a]	54.68 ± 7.04[b]	52.55 ± 8.63[b]
MDA	2.40 ± 0.24	2.70 ± 0.40	1.80 ± 0.17[a,b]	1.80 ± 0.35[a,b]
PCO	9.05 ± 0.25	12.24 ± 0.89[a]	8.09 ± 0.94[b]	9.45 ± 0.37[b]
PSH	42.87 ± 8.07	38.77 ± 7.65	36.72 ± 6.25	35.41 ± 6.61
NPSH	36.26 ± 3.51	36.43 ± 4.60	36.22 ± 4.18	37.16 ± 4.31

Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to clofibrate alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups in each row. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD ($n=10$). Abbreviations: *H_2O_2* hydrogen peroxide generation (μmol/mg protein), *MDA* malondialdehyde (μmol of MDA formed/mg protein), *PCO* protein carbonyl (μmol/mg protein), *PSH* protein thiol (μmol/mg protein), *NPSH* non-protein thiol (μmol/mg protein)

Haemodynamic Parameters

Results shown in Fig. 1 indicate significant (p<0.05) increase in systolic, diastolic and mean arterial pressure of rats administered with NaF compared to the control rats. Contrary to this, the significant (p<0.05) increase in systolic, diastolic and mean arterial pressure precipitated by NaF toxicity and treatment with clofibrate or lisinopril statistically reduced the aforementioned high blood pressure parameters. The blood pressure values obtained for clofibrate were comparable to those of the standard antihypertensive drug lisinopril (Fig. 1). This is suggestive of the possible antihypertensive effect of clofibrate.

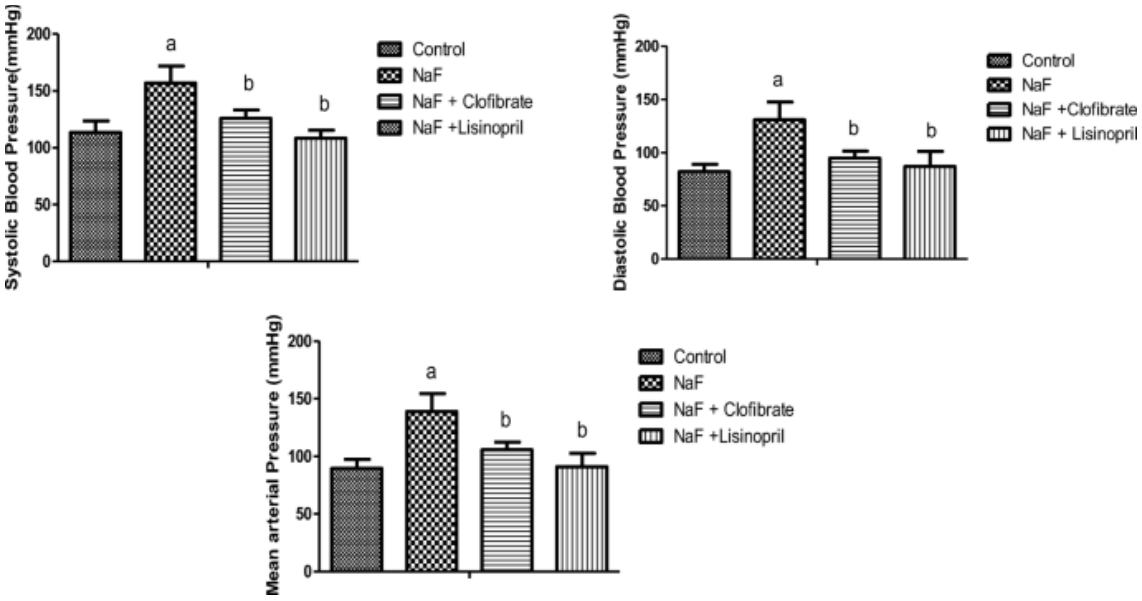

Fig. 1. Effect of clofibrate on systolic, diastolic and arterial pressure in sodium fluoride (NaF)–induced hypertension. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at p<0.05 (n= 10). Superscripts denote statistical difference across all groups. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD

Serum Nitric Oxide Bioavailability and Kidney Function Tests

The serum nitric oxide (NO) production values were found to reduce (p<0.05) significantly in NaF-induced intoxicated rats (Fig. 2). However, the administration of clofibrate or lisinopril caused significant improvement in the production of serum NO compared to NaF alone or the control rats (Fig. 2). As observed in this study, NaF intoxication precipitated a significant (p<0.05) increase in blood urea nitrogen (BUN) and creatinine of NaF alone (Fig. 2). The observed increase in values of renal function tests (BUN and creatinine) was reduced to near control values in rats treated with clofibrate (Fig. 2).

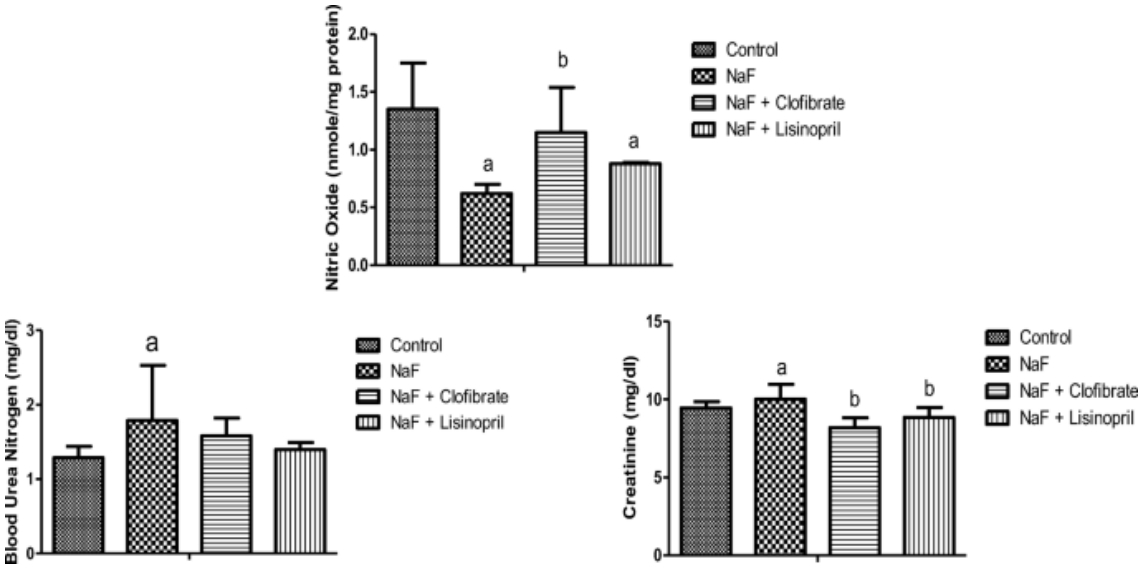

Fig. 2. Effect of clofibrate on serum renal function tests and nitric oxide in sodium fluoride (NaF)–induced hypertension. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD

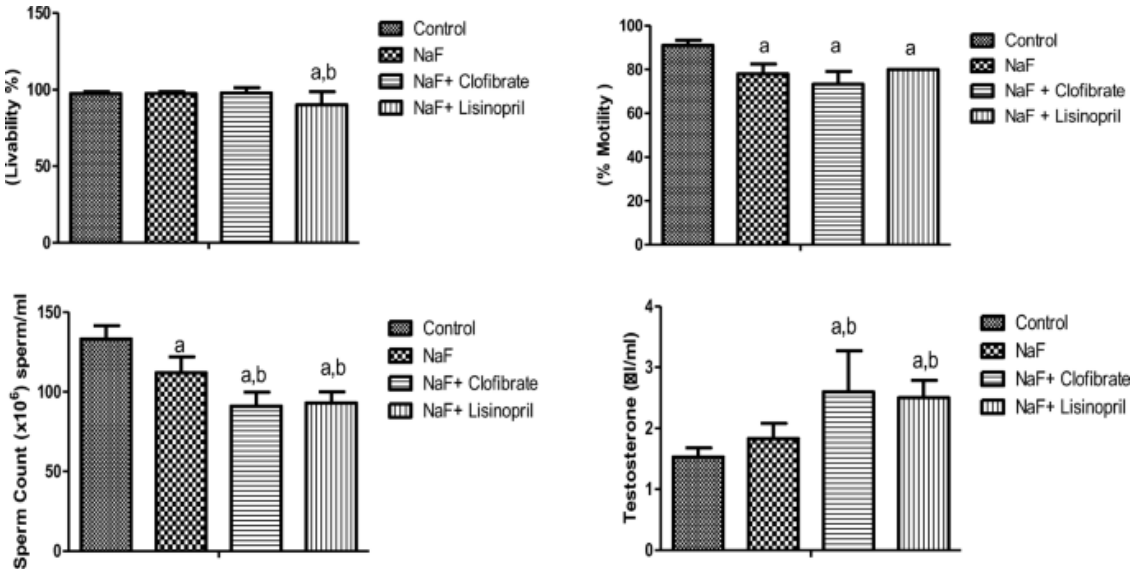

Fig. 3. Effect of clofibrate on semen characteristics and serum testosterone levels in sodium fluoride (NaF)–induced hypertension. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD

Spermiogram and Serum Testosterone Levels

The results from the spermiogram revealed significant (p<0.05) reduction in percentage (%) sperm counts and (%) sperm motility in NaF-intoxicated rats and rats treated with clofibrate or lisinopril (Fig. 3). The values of (%) sperm livability were also found to reduce in rats treated with lisinopril in comparison to the control and rats administered with NaF (Fig. 3). Combining all, neither clofibrate nor lisinopril was able to restore the sperm cell characteristics to near normal values as presented in Fig. 3. In another experiment, the values for the serum testosterone indicated significant (p<0.05) improvement in rats treated with clofibrate (Fig. 3). From the present result, slight increase in serum testosterone levels (not significant) was observed.

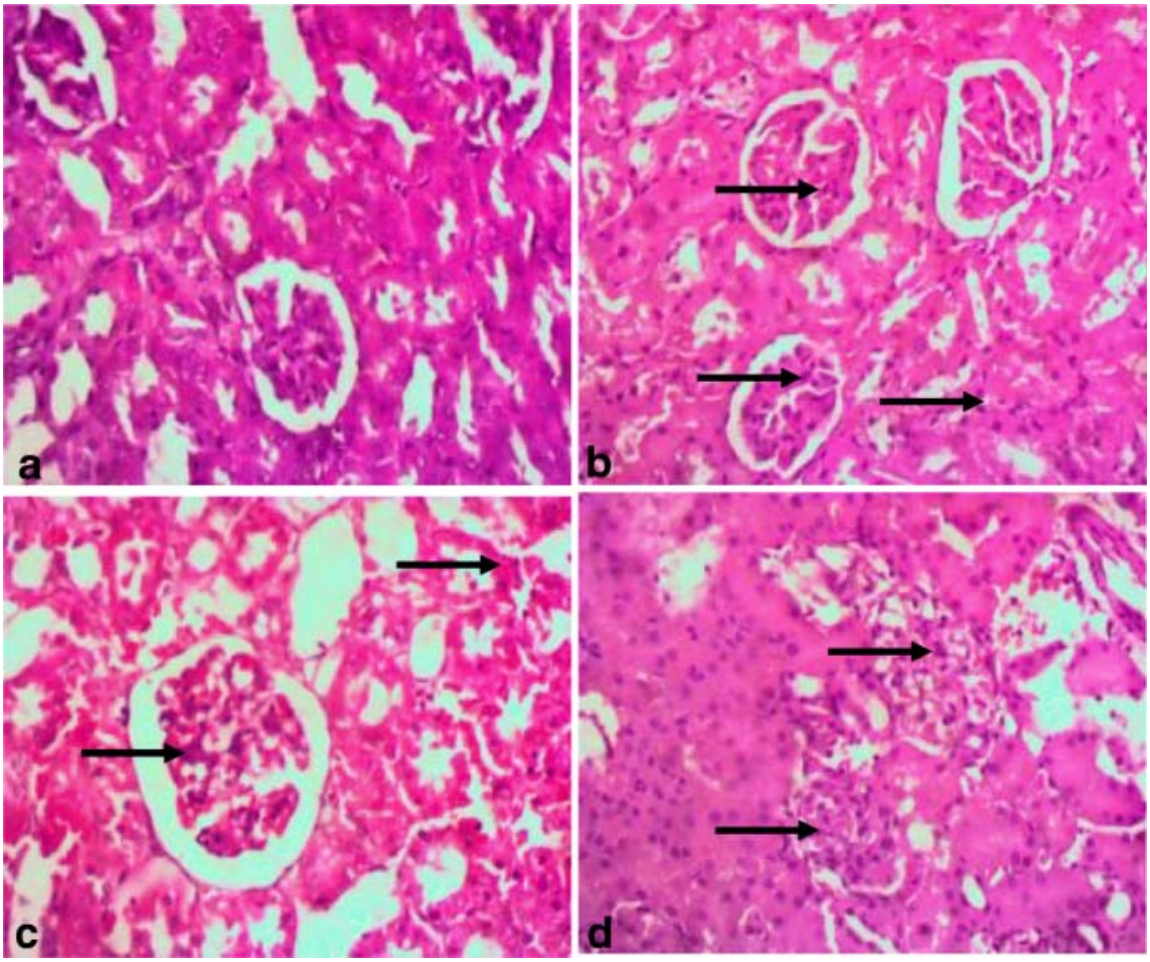

Fig. 4. Histology of the kidney showing the effect of sodium fluoride–induced hypertension in Wistar rats. **a** (Control) shows no observable lesion. **b** (NaF; 300 ppm) there is moderate thickening of the glomerular wall and mesangium (black arrows), attenuation of tubular epithelial lining and luminal ectasia (black arrows). **c** (NaF + clofibrate 100 mg/kg) there was moderate congestion of glomerular capillaries (black arrows), attenuation and degeneration of tubular epithelial lining and luminal ectasia (black arrows). **d** (NaF + lisinopril 10 mg/kg) there was regeneration of tubular epithelial cells (arrows) and a few ectatic tubular lumen. Stained with haematoxylin and eosin (H&E) mag. ×400

Histopathology

The histopathology of renal tissues showed moderate thickening of the glomerular wall and mesangium together with attenuation of epithelial lining and luminal ectasia in hypertensive rats, while moderate tubular congestion and regeneration of tubular epithelial cells were found in hypertensive rats treated with clofibrate or lisinopril, respectively (Fig. 4).

Immunohistochemistry

The immunolocalization of angiotensin II receptor type 1 (AT1 receptor) showed higher renal expression of the AT1 receptor in NaF-intoxicated rats compared to control rats (Fig. 5). However, lower expressions of the AT1 receptor were obtained in rats treated with clofibrate or lisinopril relative to the NaF alone group (Fig. 5). In Fig. 6, the NF-κB expressions increased (p<0.05) significantly across all groups, with NaF alone rats showing higher expressions of NF-κB relative to the control and rats treated with either clofibrate or lisinopril (Fig. 6). In Fig. 7, renal ACE was markedly expressed in hypertensive rats compared to control rats. However, the antihypertensive effect of clofibrate was demonstrated with significant (p<0.05) reduction in the expressions of ACE in rats treated with clofibrate when compared to untreated rats (Fig. 7). However, significant reduction in expression of ACE was obtained in rats treated with lisinopril as indicated in Fig. 8. Another receptor of note in this study is mineralocorticoid receptor (MCR). In the present research, higher expression of MCR was recorded in NaF alone group (Fig. 8). However, there was a reduction in the expression of MCR in treated rats, although not statistically significant when compared to the control rats. This indicates that clofibrate might not be using the MCR pathway to reduce high blood pressure. Lastly, testicular caspase 3 was assessed in this study to explore possible reproductive toxicity that might occur in NaF toxicity. In Fig. 9, higher expressions of testicular caspase 3 were observed in NaF-intoxicated rats and rats administered with 250 mg/kg of clofibrate when compared to the control. Interestingly, concurrent administration of lisinopril caused significant (p<0.05) reduction in the expression of caspase 3 compared to NaF alone and the control (Fig. 9).

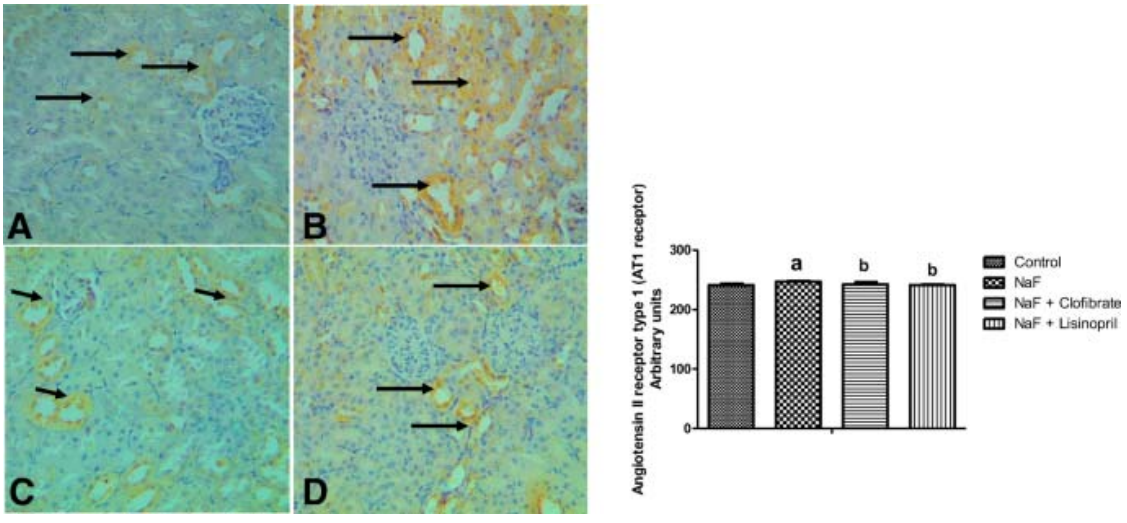

Fig. 5. The immunohistochemistry of angiotensin II receptor type 1(AT1 receptor) in sodium fluoride (NaF)–induced hypertension. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at p<0.05 (n= 10). NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD. Stained with high-definition haematoxylin (mag. ×400)

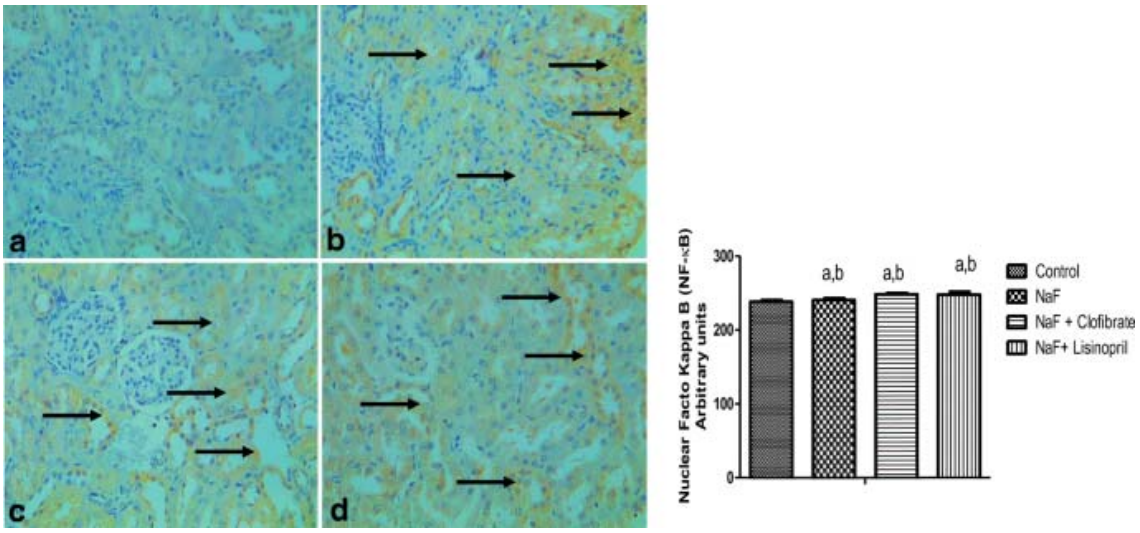

Fig. 6. The immunohistochemistry of nuclear factor kappa beta receptor in sodium fluoride (NaF)–induced hypertension. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD. Stained with high-definition haematoxylin (mag. ×400)

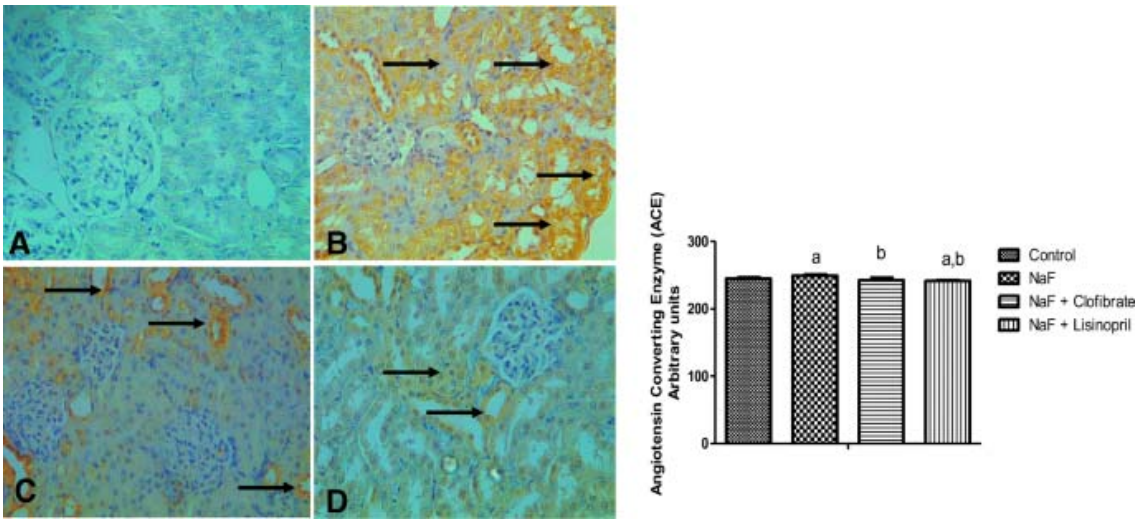

Fig. 7. The immunohistochemistry of angiotensin-converting enzyme on NaF-induced renal damage in Wistar rats. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD. Stained with high-definition haematoxylin (mag. ×400)

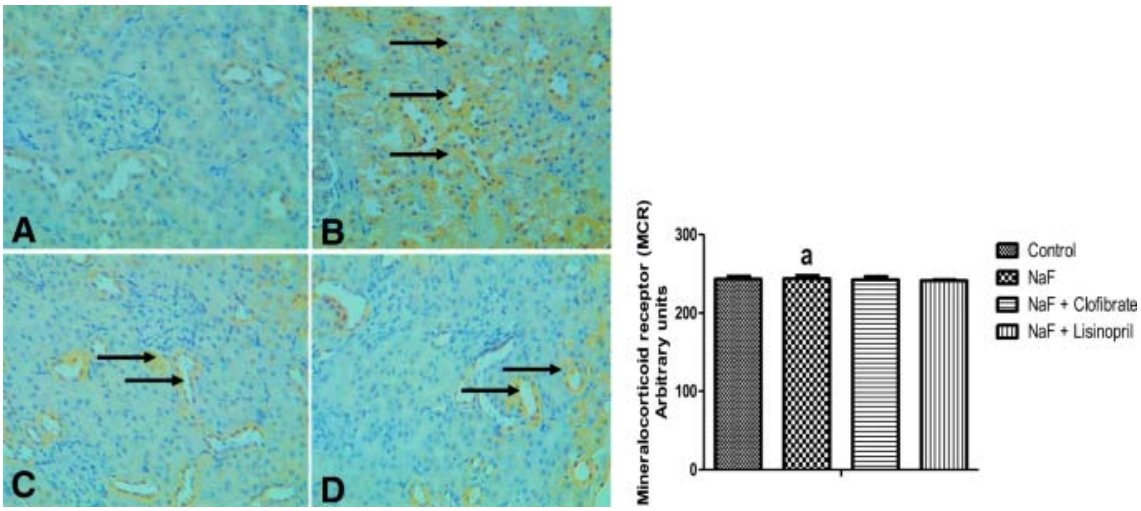

Fig. 8. The immunohistochemistry of mineralocorticoid receptor in sodium fluoride (NaF)–induced hypertension. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Superscript (a) indicates significant difference when compared to the control at $p<0.05$ ($n= 10$). Values are presented as mean ± SD. Stained with high-definition haematoxylin (mag. ×400)

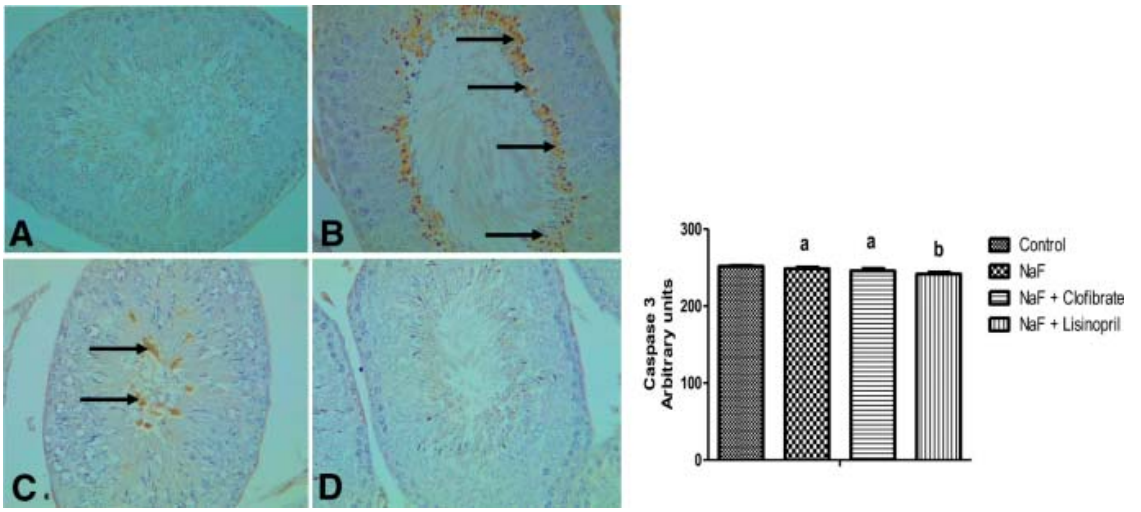

Fig. 9. The immunohistochemistry of caspase 3 in sodium fluoride–induced testicular damage. Group A (control), group B (NaF), group C (NaF + clofibrate) and group D (NaF + lisinopril). Superscript (a) indicates significant difference when compared to the control, while (b) indicates significant difference when compared to NaF alone at $p<0.05$ ($n= 10$). Superscripts denote statistical difference across all groups. NaF (300 ppm), clofibrate (250 mg/kg), lisinopril (10 mg/kg). Values are presented as mean ± SD. Stained with high-definition haematoxylin (mag. ×400)

Discussion

This study explored the use of clofibrate, a PPAR alpha agonist, in the treatment and management of NaF-induced hypertension and its complications. Clofibrate has been shown to have some pharmacological and biological activities such as anti-inflammatory, hepatoprotective, renoprotective, antioxidant and anti-atherogenic [33, 53, 91, 93, 101]. However, research reports on the antihypertensive effect of clofibrate are very scarce. Hence, the current research hypothesized that clofibrate has antihypertensive and protective effect

against reproductive toxicity associated with NaF-induced toxicity. Furthermore, we explored the potential mechanism of action of clofibrate vis-a-vis its antihypertensive activity through the renin-angiotensin-aldosterone system (RAAS) and inhibition of angiotensin-converting enzyme.

Previous studies from our laboratory established the use of NaF for the induction of hypertension in experimental rat models [64,65,66,67]. From the present study, NaF intoxication precipitated hypertension as indicated with significant increases in systolic, diastolic and mean arterial blood pressure. However, concurrent administration of clofibrate to rats that received only NaF normalized the systolic, diastolic and mean arterial blood pressures compared to that of control rats. Interestingly, the result obtained from hypertensive rats treated with clofibrate was comparable to that of ACE inhibitor lisinopril. This remarkable reduction in high blood pressure was outstanding in this study. Therefore, we can speculate that clofibrate could be repurposed from a cholesterol-lowering drug to an antihypertensive agent.

Nitric oxide (NO) is another signaling molecule of which its bioavailability has been negatively correlated with hypertension, indicating that lower NO production might contribute to the development of hypertension later in life [16, 32, 52]. From our results, NaF toxicity led to a significant depletion of serum NO production, suggesting impairment of NO functions and signalling in NaF intoxication. More so, that impairment of NO could enhance endothelial dysfunction, arterial stiffness, arteriosclerosis, platelet aggregation and thrombi formation and increase peripheral resistance, culminating in the development of hypertension [15, 29, 92].

Oxidative stress, which is the imbalance between generation of reactive oxygen species (ROS) and the antioxidant defence system in favour of ROS production, has been linked to the pathophysiology of cardiovascular diseases, with hypertension being one of the risk factors [18, 19, 83]. Apart from the established involvement of ROS in cardiovascular diseases, ROS has been shown to damage DNA, lipids and protein in biological systems [36, 45]. The exaggerated production of lipid peroxidation products such as malondialdehyde (MDA), protein carbonyl and hydrogen peroxide (H_2O_2) could be inferred from the participation of NaF ions in free radical generation and depletion of in vivo antioxidant system. The increase in content of MDA in rats that received NaF was indicative of peroxidation of polyunsaturated fatty acids of the biological membranes, as MDA is a product of lipid peroxidation [87]. Protein carbonylation is one of the most deleterious irreversible oxidative protein modifications in biological systems [24]. It has been considered as a major hallmark of oxidative stress–related disorders such as Alzheimer's disease (AD), rheumatoid arthritis, diabetes, sepsis, chronic renal failure, respiratory distress syndrome and hypertension [10, 24, 68]. Our study therefore demonstrated that NaF toxicity enhanced renal and testicular lipid peroxidation and protein carbonylation coupled oxidative stress. The observed oxidative stress was also coupled with remarkable depletion of GSH, a non-enzymic intracellular antioxidant defence system. It is worth to note that significant reduction in vitamin C content was observed in testicular tissue, while renal vitamin C content improved significantly in NaF-treated rats. This is indicative of ability of renal tissues to cope with oxidative stress precipitated by NaF or probably de novo synthesis of renal GSH. However, rats treated with clofibrate had a reduction in the values of oxidative stress markers similar to the control rats and comparable to rats administered lisinopril.

As NaF administration induced oxidative stress, the activities of the systemic antioxidant defence system were also severely inhibited. This was demonstrated as a reduction in the SOD, GPx and GST activities in NaF-intoxicated rats. It is significant to note that the activities of

these antioxidant enzymes became improved in rats co-administered either clofibrate or lisinopril, indicating the antioxidant activity of clofibrate. In the antioxidant enzyme hierarchy, SOD is first in the line of defence, where it participates in the dismutation of the superoxide anion radical to H_2O_2 [96]. The H_2O_2 generated and other toxic hydroperoxides can be further detoxified by GPx and phase 2 enzyme, GST to water and molecular oxygen [41]. When these enzymes are inhibited, accumulation of free radicals and reactive electrophiles become inevitable. The implication of this is that, apart from the observed high blood pressure precipitated by NaF, complications such as renal damage and reproductive failure might ensue as observed in the present study. We can infer from the above that inhibition of SOD activity could impair NO production as superoxide anion radicals accumulate; it binds to NO with concomitant generation of peroxynitrite ($ONOO^-$), a cytotoxic signalling molecule [42]. The generated $ONOO^-$ could also uncouple endothelial nitric oxide synthase (eNOS), leading to production of free radicals instead of NO [79]. The uncoupling of eNOS has been found to enhance endothelial dysfunction and promote oxidative and nitrosative stress [7, 26, 85]. From this study, we observed renal damage and reproductive failure as complications from NaF-induced toxicity. The values of kidney function tests (BUN and creatinine) increased significantly in intoxicated rats compared to control rats. The BUN and creatinine have been used as biomarkers of kidney damage for more than 4 decades; however, the sensitivity and specificity are considered to be low as they are influenced by many renal and nonrenal factors independent of kidney function [31, 49]. In renal injury, when glomerular filtration and renal clearance is compromised following renal damage, more of BUN and creatinine escape into the blood, and as such, they are used as renal function tests [84]. However, serum creatinine and urinary creatinine clearance have been shown to be more specific as markers of renal damage than BUN, as glomerular filtration rate is the best overall measure of kidney function [70]. More so, other highly sensitive biomarkers of acute kidney damage with incredible precision and sensitivity have been developed, namely kidney injury molecule 1 (Kim-1), neutrophil gelatinase–associated lipocalin (NGAL), cystatin C, epidermal growth factor (EGF) and osteopontin (OPN) [11, 81]. The enhancement in free radical generation and depletion of antioxidant systems as indicated by exaggerated renal oxidative stress by NaF intoxication might have contributed significantly to observed renal damage which was positively correlated with significant increases in the serum BUN and creatinine [2, 46]. Hence, NaF toxicity could therefore precipitate reduced glomerular filtration rate and renal creatinine clearance, glomerulonephritis and glomerulopathy. Parallel to this, rats treated with clofibrate had lower serum BUN and creatinine, indicating the nephroprotective effect of clofibrate, and clofibrate could help improve renal function and glomerular filtration rate.

The renin-angiotensin-aldosterone system (RAAS) and mineralocorticoid receptor inactivation has become a molecular therapeutic target in treating hypertension associated with cardiovascular and renal diseases [5, 61, 77, 94]. In our study, the immunohistochemistry showed higher expression of the renal AT1R in NaF group, while lower expression of AT1R was observed in rats treated with clofibrate. Downregulation of the AT1R in rats treated with clofibrate might be suggestive of modulation of AT1R signalling by clofibrate. Zhu et al. [100] reported that renal renin-angiotensin-aldosterone system activation in salt-sensitive hypertensive rats enhanced aldosterone production and increased ACE activity, renin production and AT1R activity. Clofibrate has been reported to decrease angiotensin II, AT1 receptor and ACE expressions, improve endothelial nitric oxide synthase participation and enhance antioxidant defence system [40]. Yousefipour and Newaz [97] also document improvement in nitric oxide production as a mechanism of antihypertensive action of clofibrate.

Therefore, hypertension induced by NaF toxicity could be said to support an increase in AT1R activity, renin and aldosterone production and hence hypertension. Quantitatively, rats treated with clofibrate showed increased renal AT1R expressions; however, it was not statistically significant when compared with the control and untreated rats. Therefore, blockage of AT1R might be the mechanism of antihypertensive effect of clofibrate. Similarly, the expressions of ACE increased significantly in NaF-induced toxicity rats.

The NF-κB is known for regulation of numerous genes that are involved in survival, inflammation and immune responses [74]. The NF-κB has been documented to participate in many cellular processes including cell growth and proliferation, metabolism, apoptosis, immune responses, and differentiation [13]. The previous study from our laboratory confirmed the involvement of NF-κB in NaF-induced hypertension [64]. Activation of NF-κB signalling in hypertensive rats has been reported to enhance the exaggeration of oxidative stress and inflammation [23, 63]. Considering the levels of observed oxidative stress in the NaF-intoxicated rats, it is not unlikely that NaF intoxication might not have switched on in NF-κB signalling. Again, higher expressions of NF-κB were observed in NaF-untreated rats. Unfortunately, treatment of rats with clofibrate could not restore the NF-κB level to that of the control. The thickening of the glomerular wall observed at the histology level also attests to the activation of NF-κB in NaF-induced toxicity. We speculate that the observed inflammatory response in the treated rats would have been normalized if the duration of experiment was more than 7 days. Hence, there is a need to explore the involvement of NF-κB signalling following chronic exposure to NaF toxicity in our future study.

The ACE is the enzyme that converts angiotensin I to a potent vasoconstrictor, angiotensin II (Ang II) [89]. Therefore, pharmacological inhibitors of ACE such as lisinopril have been reported to have both antihypertensive and renoprotective properties [61]. In this study, significant inhibition of renal ACE was observed as indicated by lower expressions of renal ACE in rats treated with clofibrate, which was comparable to lower expressions of ACE recorded for lisinopril. Pharmacologically, mineralocorticoid receptor over-activation has been linked to the development of hypertension and chronic kidney disease [4, 35, 56]. It was evident from our study that NaF intoxication enhanced over-activation of the mineralocorticoid receptor as observed from the hypertensive rats with higher expressions of mineralocorticoid receptor. The mineralocorticoid receptor expressions, however, in rats treated with clofibrate were similar to those of control and rats treated with lisinopril. Our study is therefore in support of previous studies linking over stimulation of the mineralocorticoid receptor and hypertension [4, 35]. Combining the activation of AT1R activity and the ACE and mineralocorticoid receptors by NaF could enhance production of aldosterone, facilitate sodium retention and potassium loss and ultimately induce hypertension.

The present study shows that rats with NaF intoxication have appreciable reduction in percentage (%) sperm counts and motility in comparison to the control. Surprisingly, however, treatment of rats with either clofibrate or lisinopril could not restore NaF toxicity to normal sperm characteristics. Previous research findings reported an association between NaF intoxication, testicular oxidative stress, apoptosis and autophagy [3, 25, 38, 98, 99]. Also, some findings have associated high blood pressure and testicular damage and infertility in spontaneously hypertensive rats [56]. Our findings are therefore in agreement with the aforementioned reports on NaF and testicular toxicity as indicated with significantly higher expressions of testicular caspase 3 in NaF-intoxicated rats relative to the control and treated rats. The anti-apoptotic property was demonstrated as treatment of rats with clofibrate significantly reduced testicular caspase 3 than in the untreated rats. This, therefore, confirms

the earlier reports of induction of apoptosis following NaF intoxication [3, 38]. Interestingly, serum testosterone levels were not affected in NaF-intoxicated rats. In fact, rats treated with either clofibrate or lisinopril had significantly higher levels of testosterone than the control rats. We therefore speculate that short-term exposure to clofibrate might have androgenic effects due to the observed increase in serum testosterone levels in NaF-intoxicated rats.

Conclusion

In conclusion, NaF intoxication caused increased high blood pressure parameters (systolic, diastolic and mean arterial pressure), oxidative stress, depletion of antioxidant defence system, reduced nitric oxide production, activation of angiotensin-converting enzyme activity, activation of angiotensin II type 1 receptor, mineralocorticoid receptor over-activation and activation of NF-κB. Further, renal and testicular damage was also observed in NaF-intoxicated rats. However, treatment of intoxicated rats with clofibrate led to reduction in renal and testicular oxidative stress, improved antioxidant status and lowered high blood pressure through the inhibition of angiotensin-converting enzyme activity, angiotensin II type 1 receptor and mineralocorticoid receptor over-activation. Overall, from the present study, the mechanism of action of antihypertensive effect of clofibrate was mediated through inhibition of angiotensin-converting enzyme, angiotensin II type 1 receptor and mineralocorticoid receptor and improvement in nitric oxide bioavailability with concomitant reduction of high blood pressure. Hence, clofibrate could be repurposed as an antihypertensive agent for the management of hypertension.

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Ethics declarations

Ethics Approval

All animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). The Animal Care and Use Research Ethical Committee of the Faculty of Veterinary Medicine of Ibadan approved the study with ethical approval number UI-ACUREC/19/124.

Consent to Participate

Not applicable

Consent for Publication

Not applicable

Conflicts of Interest

The authors declare no competing interests.

Availability of Data and Materials

Data will be made available based on request from the corresponding author.

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