



Original article

Estrogen-progestin oral contraceptive and nicotine exposure synergistically confers cardio-renoprotection in female Wistar rats



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ARTICLE INFO

Keywords:

Estrogen-progestin therapy
Cardiac Na^+/K^+ -ATPase activity
Lipid
Insulin resistance
Metabolic stress
Nicotine

ABSTRACT

Approximately fifty percent of premenopausal women who smoke cigarettes or on nicotine replacement therapy are also on hormonal contraceptives, especially oral estrogen-progestin. Oral estrogen-progestin therapy has been reported to promote insulin resistance (IR) which causes lipid influx into non-adipose tissue and impairs Na^+/K^+ -ATPase activity, especially in the heart and kidney. However, the effects of nicotine on excess lipid and altered Na^+/K^+ -ATPase activity associated with the use of estrogen-progestin therapy have not been fully elucidated. This study therefore aimed at investigating the effect of nicotine on cardiac and renal lipid influx and Na^+/K^+ -ATPase activity during estrogen-progestin therapy. Twenty-four female Wistar rats grouped into 4 ($n = 6/\text{group}$) received (*p.o.*) vehicle, nicotine (1.0 mg/kg) with or without estrogen-progestin steroids (1.0 μg ethinyl estradiol and 5.0 μg levonorgestrel) and estrogen-progestin only daily for 6 weeks. Data showed that estrogen-progestin treatment or nicotine exposure caused IR, hyperinsulinemia, increased cardiac and renal uric acid, malondialdehyde, triglyceride, glycogen synthase kinase-3, plasminogen activator inhibitor-1, reduced bilirubin and circulating estradiol. Estrogen-progestin treatment led to decreased cardiac Na^+/K^+ -ATPase activity while nicotine did not alter Na^+/K^+ -ATPase activity but increased plasma and tissue cotinine. Renal Na^+/K^+ -ATPase activity was not altered by the treatments. However, all these alterations were reversed following combined administration of oral estrogen-progestin therapy and nicotine. The present study therefore demonstrates that oral estrogen-progestin therapy and nicotine exposure synergistically prevents IR-linked cardio-renal toxicity with corresponding improvement in cardiac and renal lipid accumulation, oxidative stress, inflammation and Na^+/K^+ -ATPase activity.

1. Introduction

Cardiac and renal metabolic disorders are interconnected and are responsible for mortality and morbidity globally. Cardiorenal fat accumulation and insulin resistance (IR) have been implicated in the pathogenesis of cardiac and renal dysfunctions in type 2 diabetic patients. Myocardial and renal fat accumulation has been reported in obese and type 2 diabetic patients and it has been associated with left ventricular dysfunction, cardiac hypertrophy, fibrosis [1,2], glomerulosclerosis, tubulointerstitial injury, podocyte dysfunction and apoptosis [3,4]. Triglyceride (TG) is the body's dominant energy storage form, serving as a storehouse for and supply of free fatty acids (FFAs) for mitochondrial beta oxidation and consequently, adenosine triphosphate production [5]. Increased myocardial and renal TG accumulation may reflect a mismatch between the uptake and oxidation of fatty

acids. Hence, lipotoxicity and/or TG accumulation contributes to the pathogenesis of cardiorenal dysfunction [2,3].

Dyslipidemia is a reversible risk factor involved in the pathogenesis of kidney disease and consequent cardiovascular mortality [6,7]. Increased ectopic lipid storage in the skeletal muscle and liver is also associated with IR [8]. Studies have documented that intracellular lipid accumulation in ectopic tissues contributes to the development of impaired insulin signaling. Therefore, the development of IR is strongly associated with lipid accumulation in sites other than adipose tissue [9] such as heart and kidney resulting in cardiorenal lipotoxicity and/or dysfunction.

Na^+/K^+ -ATPase is an energy-transducing ion pump present in the plasma membrane of most mammals [10]. Besides the maintenance of electrochemical gradient through the transportation of three Na^+ out of the cell and two K^+ into the cell [11], Na^+/K^+ -ATPase also regulates

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<https://doi.org/10.1016/j.bioph.2020.110387>

Received 12 April 2020; Received in revised form 4 June 2020; Accepted 7 June 2020

Available online 12 June 2020

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protein tyrosine phosphorylation resulting in cellular signal transduction [12]. Studies have demonstrated the association of impaired Na^+/K^+ -ATPase activity with metabolic, cardiac and renal dysfunctions [13,14].

Hyperuricemia has been implicated in the pathogenesis of cardiovascular, renal and metabolic diseases [15,16]. Furthermore, a recent study demonstrated the involvement of myocardial uric acid (UA) in the cause of cardiac damage [17]. Likewise, UA was reported to induce the phenotypic transition of renal tubular cells thereby causing chronic kidney disease [18]. The pro-oxidant nature of UA when it gains entry into the intracellular sites may be responsible for UA-associated cardiac and renal tissue damage [19]. Furthermore, UA accumulation is associated with endothelial dysfunction due to the reduction of nitric oxide bioavailability. In addition, elevated UA level has been linked to increased lipid oxidation and hypertriglyceridemia [20].

Nicotine is a stimulant responsible for the addictive nature of tobacco, hence its extensive use in nicotine replacement therapy (NRT) as nicotine gum, patch or intranasal preparations for smoking cessation [21]. It was shown that women are more susceptible to nicotine dependence than men since they have greater nicotine and cotinine clearance because of the sex hormone, estrogen [22]. Also, among women of reproductive age, pregnancy and oral contraceptives use increase nicotine and cotinine clearance [22]. Nevertheless, studies have demonstrated that long-term usage of nicotine gum causes IR and hyperinsulinemia. In contrast, nicotine has been shown to have beneficial effects in diseases with an inflammation-mediated mechanism such as experimental type 1 diabetes, ulcerative colitis, and pre-eclampsia [23–25].

Oral hormonal contraception remains one of the most effective reversible contraceptive methods commonly used worldwide. The adverse contribution of combined oral contraceptives (COC) to the pathogenesis of cardiometabolic disorders remains unsettled despite the newer generations of COC [26]. Large percentages of reproductive-aged women who smoke cigarettes also use hormonal contraceptives sometimes in their lives. Furthermore, COC is the most common type of hormonal contraception currently in use [27]. Regardless of the popularity of hormonal contraceptives usage among premenopausal women who smoke concomitantly or on NRT, there is a dearth of information on the effects of hormonal contraceptives on non-smoking nicotine exposure. This study was designed to elucidate the potential role of non-smoking nicotine exposure on cardiorenal lipid influx and Na^+/K^+ -ATPase activity in oral estrogen-progestin-treated female rats.

2. Materials and method

2.1. Animals and treatments

All experimental protocols were approved by the University of Ilorin Ethical Review Committee (UERC) following guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and every effort was made to minimize both the number of animals used and their suffering. Ten weeks old female Wistar rats were used for the study. The animals were housed in a well-ventilated room maintained under standard conditions of light, feeding and temperature. Rats had unrestricted access to standard rat chow and tap water. After one week of acclimatization, the animals were randomly assigned to one of the following experimental groups ($n = 6/\text{group}$) of comparable body weight: Control group received distilled water (vehicle; *p.o*) daily, NIC-treated group received (*p.o*) nicotine (1.0 mg/kg), COC-treated group received (*p.o*) a combination of 1.0 mg ethinylestradiol and 5.0 mg levonorgestrel while COC + NIC-treated group received combination of 1.0 mg ethinylestradiol and 5.0 mg levonorgestrel plus 1.0 mg/kg nicotine daily. All drugs and vehicle were given daily by oral gavage syringe for six weeks.

2.2. Sample preparation

At the end of treatment, the rats were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*) after 12 h overnight fast. Blood was collected by cardiac puncture into heparinized bottles. The blood was centrifuged at 3000 rpm for 5 min. Plasma was stored frozen until needed for biochemical assay. The heart and kidneys were removed, cleared of adhering connective tissues, blotted and weighed immediately. After weighing, 100 mg of each tissue was carefully removed, homogenized in cold phosphate-buffered solution with a glass homogenizer and centrifuged at 10,000 rpm for 10 min at 4 °C.

2.3. Fasting glucose and insulin resistance

Fasting blood glucose level was determined with a glucometer (ONETOUCH® -LifeScan, Inc., Milpitas, CA). The IR was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR). HOMA-IR is expressed as fasting glucose (mmol/l)*fasting insulin (IU/l)/22.5. Triglyceride-glucose index (TyG), a surrogate of IR was estimated using $\text{Ln}(\text{TG}(\text{mg/dl}) \times \text{FBG}(\text{mg/dl})/2)$ [28].

2.4. Visceral adiposity

Following blood sample collection, all intra-abdominal fat depots including perirenal, urogenital, and retroperitoneal were isolated, weighed and corrected to body weight to eliminate variability using an electronic weighing scale.

2.5. Na^+/K^+ -ATPase activity assay

Cardiac and renal tissue homogenates were used for Na^+/K^+ -ATPase activity determination. Na^+/K^+ -ATPase activity is an enzymatic assay that involves two similar phases; one for the estimation of total ATPase activity (incubation medium: 2 mM ATP, 100 mM NaCl, 5 mM KCl, 10 mM MgCl_2 , 1 mM EGTA, 50 mM Tris-HCl pH 7.4) and another for the assessment of ouabain insensitive ATPase activity by selectively blocking Na^+/K^+ -ATPase (incubation medium: 2 mM ATP, 1 mM Ouabain, 15 mM NaCl, 10 mM MgCl_2 , 90 mM *N*-methyl-D-glucamine chloride, 1 mM EGTA, 50 mM Tris-HCl pH 7.4). The last medium permitted the complete inhibition of Na^+/K^+ -ATPase, preserving the osmolarity. The tissue homogenates were washed with corresponding incubation media. After washing, 100 μl of enzymatic assay media were added to each well, and homogenates were incubated for 30 min in a CO_2 incubator at 37 °C. Subsequently, 60 μl of supernatant was obtained by centrifugation of the homogenates at 5000 rpm for 15 min and transferred to another microplate containing the same volume of trichloroacetic acid (TCA) 11.5%. The ATPase activity was then determined by colorimetric quantification of inorganic phosphate (Pi) formed in 30 min by using ammonium molybdate. Na^+/K^+ -ATPase activity was evaluated by the difference between the results gotten using the previously described experimental conditions, that is, in the absence and presence of ouabain [29]. Na^+/K^+ -ATPase activity was expressed as micromole of inorganic phosphate released per milligram protein per hour. Protein concentration was estimated by Biuret method using commercial kit supplied by Randox Laboratory Ltd. (Antrim, UK).

2.6. Biochemical assays

Plasma insulin and estradiol were determined using ELISA kits from Ray Biotech, Inc. (Georgia, USA) and Elabscience Biotechnology Co., Ltd., (Wuhan, China) respectively. Cardiac and renal glycogen synthase kinase-3 (GSK-3), plasminogen activator inhibitor-1 (PAI-1) and cotinine were estimated using ELISA kits from Elabscience Biotechnology Co., Ltd., Wuhan, China and Calbiotech Inc. (El Cajon, CA, USA) respectively. Also, cardiac and renal malondialdehyde (MDA), a marker

of lipid peroxidation, was measured by standardized enzymatic colorimetric method using assay kits from Oxford Biomedical Research, Inc. (Oxford, MI, USA). Cardiac and renal uric acid, bilirubin, reduced glutathione (GSH), aspartate transaminase (AST), alkaline phosphatase (ALP), alanine transferase (ALT), fasting triglyceride (TG), total cholesterol and high-density lipoprotein cholesterol (HDL-C) were measured by standardized enzymatic colorimetric methods using reagents obtained from Randox Laboratory Ltd. (Antrim, UK). TG/HDL-C was expressed as an index of atherogenic dyslipidemia.

2.7. Data analysis and statistics

All data were expressed as means $\pm$ SEM. Statistical group analysis was performed with SPSS statistical software. One-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups. Bonferroni's test was used to identify the significance of pair wise comparisons of mean values among the groups. Statistically significant differences were accepted at $p < 0.05$.

3. Result

3.1. Estrogen-progestin COC and nicotine exposure improves body weight, organ weight and visceral adiposity in female rats

There was decreased body weight in NIC-exposed or COC-treated rats when compared with the control group. However, COC + NIC treatment reversed the reduced body weight when compared with the NIC-exposed or COC-treated female rats (Fig. 1a). Visceral adiposity was decreased in NIC-exposed rats whereas COC-treated rats had increased visceral adiposity when compared with the control group. COC + NIC treatment led to reduced visceral adiposity when compared with the COC-treated group (Fig. 1b). The heart and kidney weights were comparable across the treatment groups (Table 1).

3.2. Estrogen-progestin COC and nicotine exposure improves glucoregulation in female rats

Fasting glycemia was comparable across the experimental groups (Fig. 2a). NIC exposure or COC treatment led to increased fasting insulinemia which was abrogated by COC + NIC treatment when compared with COC-treated group (Fig. 2b). NIC exposure or COC treatment led to increased HOMA-IR whereas COC + NIC treatment reversed HOMA-IR to the control level (Fig. 2c). NIC exposure or COC treatment increased TyG-index which was suppressed by COC + NIC treatment (Fig. 2d).

3.3. Estrogen-progestin COC and nicotine exposure attenuates impaired cardiac and renal lipid metabolism in female rats

Nicotine exposure or COC treatment led to increased cardiac and renal triglyceride (TG) and total cholesterol (TC) accumulation when

compared with control whereas COC + NIC treatment led to reduced cardiac and renal TG accumulation but cardiac and renal TC accumulation was not reversed when compared with COC-treated group. Besides, NIC exposure led to reduced cardiac HDL-cholesterol content whereas COC treatment did not alter cardiac HDL-cholesterol. COC + NIC treatment did not alter cardiac HDL-cholesterol content. However, renal HDL-cholesterol content was reduced across the treatment group (Table 1). TG/HDL-C an index of atherogenic dyslipidemia was elevated by NIC exposure or COC treatment in cardiac and renal tissues whereas COC + NIC treatment reversed the cardiorenal TG/HDL-C level when compared with COC-treated group (Fig. 3).

3.4. Estrogen-progestin COC and nicotine exposure averts cardiac and renal oxidative injury in female rats

Nicotine exposure or COC treatment led to decreased cardiac and renal GSH and bilirubin level when compared with the control group, whereas COC + NIC treatment reversed cardiorenal GSH and bilirubin levels when compared with COC-treated group. Also, NIC exposure or COC treatment led to increased cardiac and renal MDA levels when compared with control group. However, COC + NIC treatment reversed the cardiac and renal MDA level when compared with the COC-treated group (Fig. 4).

3.5. Estrogen-progestin COC and nicotine exposure reduces cardiac and renal injury markers in female rats

Cardiac ALT activity was not altered across the experimental groups. However, renal ALT activity was increased in NIC exposed rats whereas COC or COC + NIC treatment did not alter renal ALT activity when compared with control. Furthermore, cardiac and renal AST activity was increased in NIC exposed rats but COC treatment did not affect the cardiac and renal AST activity when compared with control group. However, COC + NIC treatment led to decreased cardiac AST activity while renal AST activity was not altered in COC + NIC-treated rats when compared with COC-treated group. Besides, NIC exposure did not affect cardiac and renal ALP activity when compared with control. Likewise, COC treatment did not affect cardiac ALP activity but reduced renal ALP activity when compared with control. However, COC + NIC treatment suppressed cardiac and renal ALP activity when compared with COC-treated rats (Table 2).

3.6. Estrogen-progestin COC and nicotine exposure suppresses cardiac and renal GSK-3, PAI-1 and uric acid levels in female rats

Nicotine exposure or COC treatment led to increased cardiac and renal GSK-3, PAI-1 and uric acid levels which were reversed by COC + NIC treatment when compared with the COC-treated group (Fig. 5).

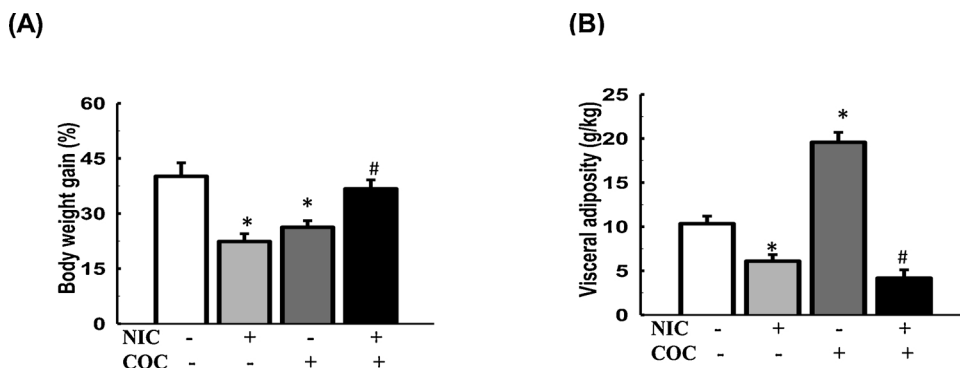


Fig. 1. COC and NIC treatment improve body weight gain (A) and reversed visceral adiposity (B) in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * $p < 0.05$ vs CON, # $p < 0.05$ vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive.

Table 1

Estrogen-progestin COC and nicotine improves cardiorenal lipid accumulation.

	CON	NIC	COC	COC + NIC
Heart (g/kg bw)	3.5 ± 0.1	3.7 ± 0.2	3.8 ± 0.1	3.9 ± 0.2
TG (mg/100 mg tissue)	99.0 ± 4.0	132.4 ± 8.3*	185.6 ± 10.7*	94.1 ± 6.1 [#]
TC (mg/100 mg tissue)	40.6 ± 9.1	146.2 ± 10.7*	92.5 ± 4.0*	98.4 ± 4.5*
HDL (mg/100 mg tissue)	88.9 ± 3.9	22.3 ± 6.0*	75.4 ± 5.1	84.3 ± 5.5
Kidney (g/kg bw)	7.8 ± 0.6	9.2 ± 0.3	9.3 ± 0.3	9.0 ± 0.3
TG (mg/100 mg tissue)	90.0 ± 2.7	113.4 ± 2.3*	132.1 ± 2.1*	101.8 ± 4.3 [#]
TC (mg/100 mg tissue)	38.4 ± 1.0	104.2 ± 2.5*	88.3 ± 6.6*	92.0 ± 4.8*
HDL (mg/100 mg tissue)	102.0 ± 4.9	56.7 ± 4.3*	70.5 ± 8.4*	83.7 ± 6.3*

Values are expressed as mean ± SEM of 6 rats per group; *p < 0.05 vs CON, #p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive, TG; triglyceride, TC; total cholesterol, HDL; high density lipoprotein cholesterol.

3.7. Estrogen-progestin COC and nicotine exposure enhances cardiac and renal Na⁺/K⁺-ATPase activity, decreases cotinine and circulating estradiol levels in female rats

Treatment with COC decreased cardiac Na⁺-K⁺-ATPase activity while NIC exposure did not alter cardiac Na⁺/K⁺-ATPase activity when compared with control group. However, COC + NIC treatment increased cardiac Na⁺/K⁺-ATPase activity when compared with both control and COC-treated groups. Also, renal Na⁺/K⁺-ATPase activity was comparable across treatment groups (Fig. 6). Furthermore, circulating estradiol was reduced across the treatment groups (Fig. 7). More so, cotinine a biomarker of NIC exposure was not detectable in the plasma, renal, and cardiac tissues of control and COC-treated groups. However, NIC exposure led to increased plasma, renal and cardiac cotinine levels when compared with control. COC + NIC treatment reduced plasma and renal cotinine levels but cotinine was not detectable in the cardiac tissue of COC + NIC-treated group (Fig. 8).

4. Discussion

The present study demonstrates that nicotine exposure or COC treatment induces cardiorenal metabolic dysfunctions as evident by increased cardiac and renal lipid, oxidative stress and inflammation.

This was preceded by IR and hyperinsulinemia despite normoglycemia in NIC-exposed or COC-treated rats. Furthermore, COC treatment in this study resulted in reduced cardiac Na⁺/K⁺-ATPase activity whereas NIC exposure on the other hand, led to increased cardiorenal AST and renal ALT activities without altering cardiac Na⁺/K⁺-ATPase activity. Renal Na⁺/K⁺-ATPase activity was preserved across the treatment group. The observed cardiorenal metabolic stress was associated with elevated uric acid and reduced circulating estradiol level. These cardiorenal alterations were synergistically counteracted by Co-administration of COC and nicotine with the exception of circulating estradiol. Therefore, the combined administration of estrogen-progestin oral contraceptive and nicotine interact synergistically to improve glucoregulation, Na⁺/K⁺-ATPase activity, reduce cardiac and renal lipid accumulation, inflammation, and oxidative stress in female rats (Fig. 9).

Hormonal contraceptives function by replacing endogenously produced ovarian hormones with synthetic analogues which have high binding affinities, thereby cutting off the hypothalamic-pituitary-gonadal feedback loop that regulates ovulation and ultimately reduces endogenous sex hormone levels and variability [30]. Estrogen-progestin oral contraceptive has been associated with the risk of developing cardiometabolic dysfunction despite its popularity among premenopausal and postmenopausal women who make use of it for different purposes such as hormonal contraception, dysmenorrheal,

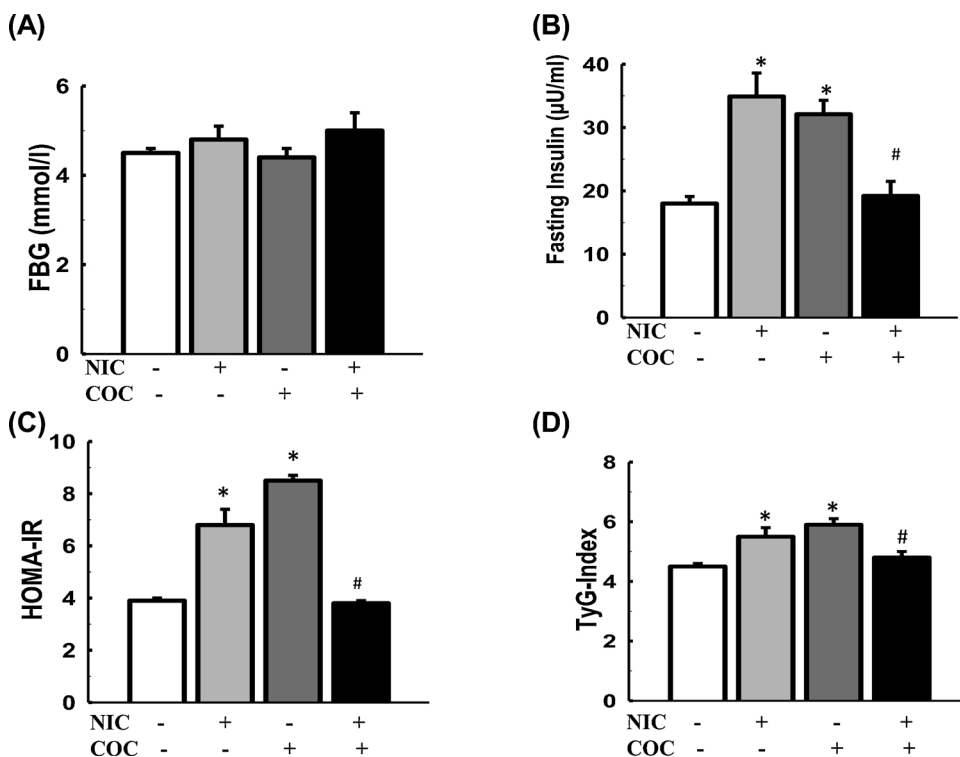


Fig. 2. COC and NIC treatment did not alter fasting blood glucose (A) but reversed fasting insulin (B), HOMA-IR and TyG-index (D) values in female Wistar rats. Values are expressed as mean ± SEM of 6 rats per group; *p < 0.05 vs CON, #p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive, TyG-index; triglyceride-glucose index.

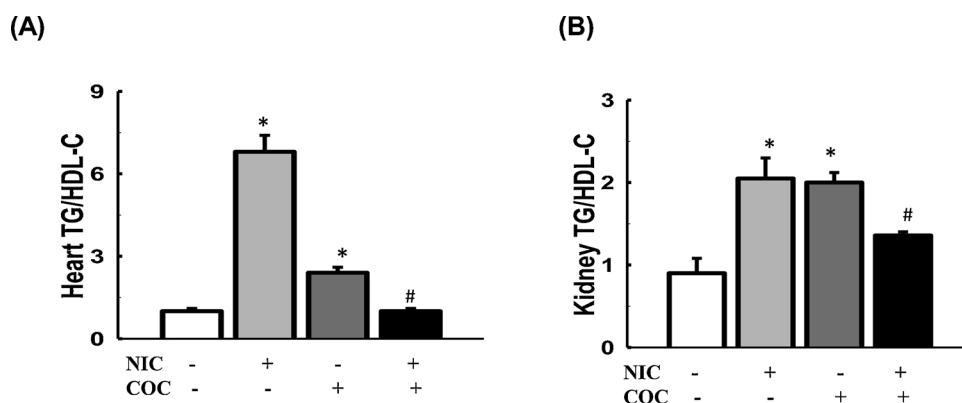


Fig. 3. COC and NIC treatment reversed cardiac and renal TG/HDL-C (A & B) in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * p < 0.05 vs CON, # p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive.

menstrual irregularities, polycystic ovarian syndrome, etc. Studies have also implicated the activation of the cardiac renin-angiotensin system, endothelial dysfunction and upregulation of intrarenal angiotensin II type 1 receptor gene in oral estrogen-progestin-induced hypertension [26,31]. Furthermore, in line with the findings of the present study, gluco-metabolic dysregulation has been associated with oral nicotine exposure or estrogen-progestin therapy [32,33]. Similarly, smoking-derived nicotine exposure has been associated with cardiometabolic complications such as IR, oxidative stress, cardiovascular and renal diseases [34]. However, there is little information on the cardiorenal metabolic effects of non-smoking nicotine exposure especially in estrogen-progestin oral contraceptive users.

The result of this study demonstrates that nicotine exposure or COC treatment result in cardiorenal lipid accumulation and increased atherogenic indices which were ameliorated by COC + NIC treatment. Therefore, nicotine or COC treatment may predispose to cardiac and renal damage through lipid accumulation. Lipid accumulation has been reported to be a crucial pathological feature in both heart and kidney

Table 2

Estrogen-progestin COC and nicotine reduces cardiac and renal injury enzymes.

	CON	NIC	COC	COC + NIC
Heart				
ALT (U/L)	60.0 $\pm$ 2.0	58.2 $\pm$ 2.3	62.3 $\pm$ 3.3	56.3 $\pm$ 2.0
AST (U/L)	61.7 $\pm$ 2.7	77.7 $\pm$ 2.2*	68.7 $\pm$ 2.0	48.7 $\pm$ 3.0*#
ALP (U/L)	4.1 $\pm$ 2.7	4.3 $\pm$ 1.0	5.5 $\pm$ 1.1	1.4 $\pm$ 0.2*#
Kidney				
ALT (U/L)	56.0 $\pm$ 2.0	68.5 $\pm$ 2.0*	59.3 $\pm$ 2.7	55.3 $\pm$ 2.0
AST (U/L)	70.0 $\pm$ 3.0	84.7 $\pm$ 2.3*	66.7 $\pm$ 3.3	69.4 $\pm$ 3.8
ALP (U/L)	5.2 $\pm$ 1.0	5.4 $\pm$ 1.8	1.9 $\pm$ 0.5*	1.1 $\pm$ 0.4*

Values are expressed as mean $\pm$ SEM of 6 rats per group; * p < 0.05 vs CON, # p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive, ALT; alanine transferase, AST; aspartate transaminase, ALP; alkaline phosphatase.

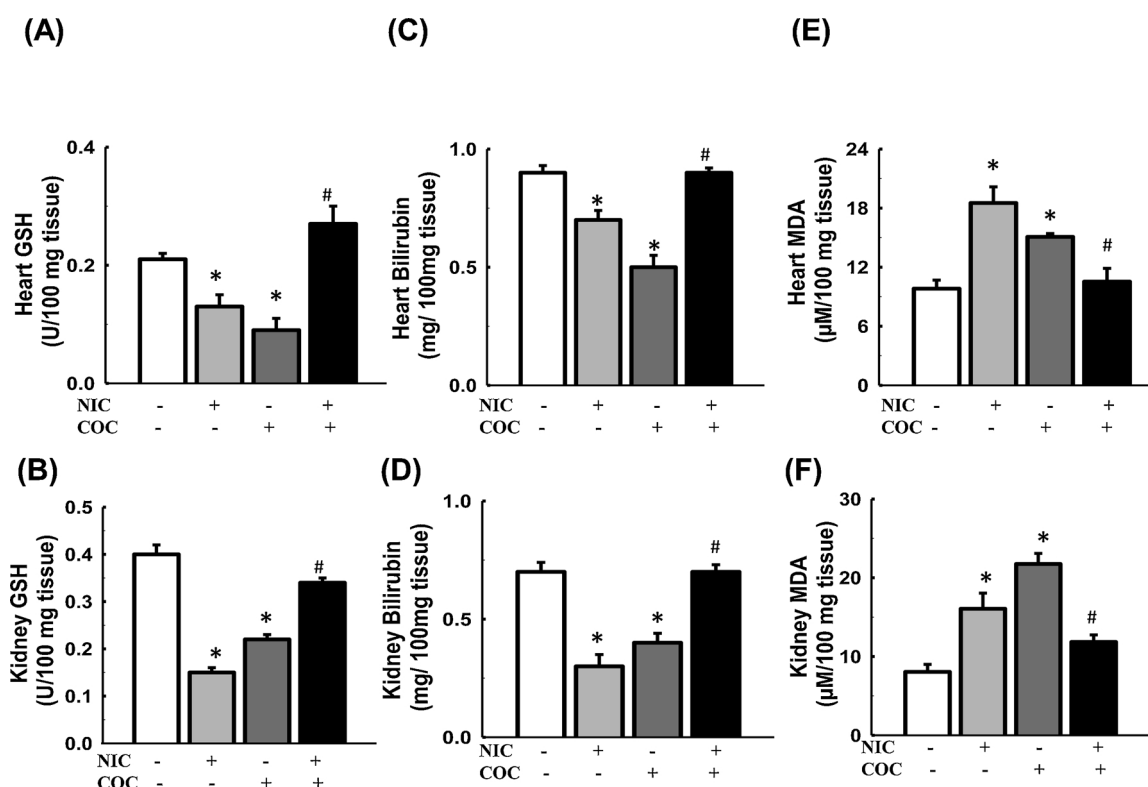


Fig. 4. COC and NIC treatment increased cardiac and renal GSH (A & B), bilirubin (C & D) and suppressed MDA (E & F) in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * p < 0.05 vs CON, # p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive, GSH; reduced glutathione, MDA; malondialdehyde.

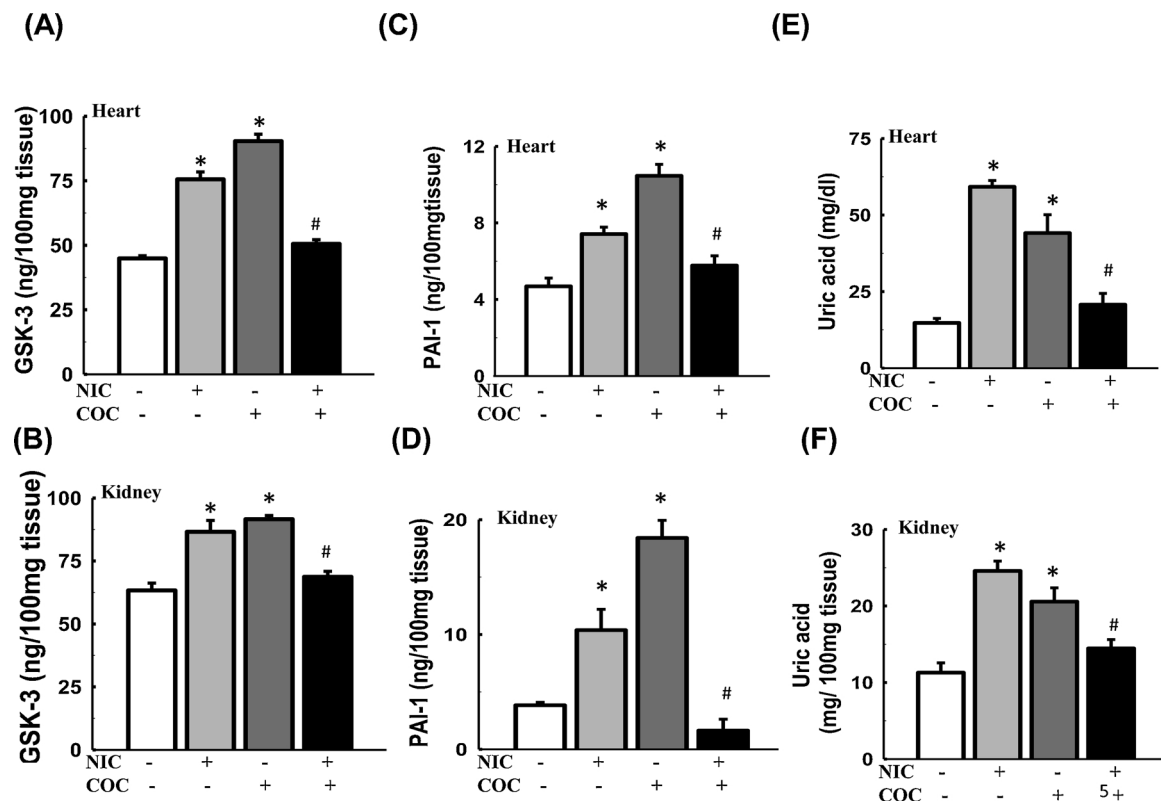


Fig. 5. COC and NIC treatment reversed cardiac and renal GSK-3 (A & B), PAI-1 (C & D) and uric acid (E & F) in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * p < 0.05 vs CON, # p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive, GSK-3; glycogen synthase kinase-3, PAI-1; plasminogen activator inhibitor-1.

diseases [2,3,35]. The entire kidney can utilize fatty acids, the cortex and the epithelial tubule cells depend on fatty acid oxidation for ATP. The cortex is also the site of large accumulations of neutral lipids during kidney disease [2]. The heart is also vulnerable to the pathological effects of lipid overload despite its preference for lipids. Hence, lipid accumulation in the heart may be responsible for the development of cardiac IR characterized by predominant utilization of fatty acid for energy resulting in mitochondrial dysfunction, endoplasmic reticulum stress, impaired intracellular energy and calcium ion metabolism [36,37]. Intriguingly, COC + NIC treatment caused reduced cardiac and renal lipid accumulation. This is buttressed by the observed decreased visceral adiposity following COC + NIC treatment in the female rats. The association of decreased lipid accumulation with amelioration of IR-linked cardiorenal metabolic dysfunctions in this animal model is noteworthy. Therefore, estrogen-progestin oral contraceptive and nicotine exposure offers benefits to counteract cardiorenal metabolic stress induced by either COC or NIC singly administration.

Furthermore, cardiorenal glutathione (GSH) and bilirubin levels

(antioxidant biomarkers) were decreased whereas cardiorenal MDA level (an index of lipid peroxidation) was elevated by NIC exposure or COC treatment. This finding implies that NIC exposure or COC treatment may be pro-oxidant in its mechanism and the outcome of poorly regulated oxidant production is excessive inflammatory response and tissue injury. The elevated MDA in the tissues (Fig. 4e, f) demonstrates that the disturbed lipid metabolism observed in the NIC exposed or COC-treated animals may cause oxidative cardiorenal damage [38,39]. Oxidative modification has been implicated in the pathogenic relationship between dyslipidemia, cardiovascular and kidney diseases [40]. The depressed cardiorenal GSH (Fig. 4a, b) and bilirubin (Fig. 4c, d) levels in the NIC-exposed or COC-treated rats indicate oxidative stress and depleted antioxidant defenses. It is important to note that, antioxidant enzymes and/or molecules (such as GSH and bilirubin) provide the foremost defense mechanism which prevents biological molecules (such as lipids, proteins) from injury by inhibiting reactive oxygen species formation, thereby preventing oxidative stress. The finding of the present study is in agreement with reports that bilirubin

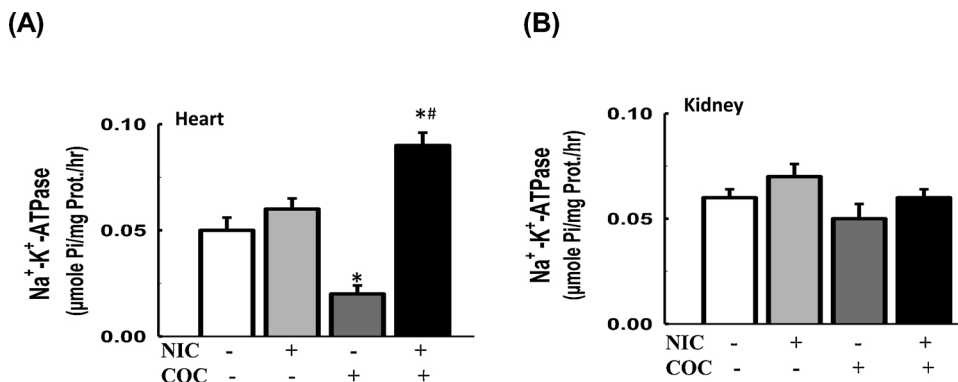


Fig. 6. COC and NIC treatment enhanced cardiac and renal Na⁺/K⁺-ATPase activity (A & B) in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * p < 0.05 vs CON, # p < 0.05 vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive.

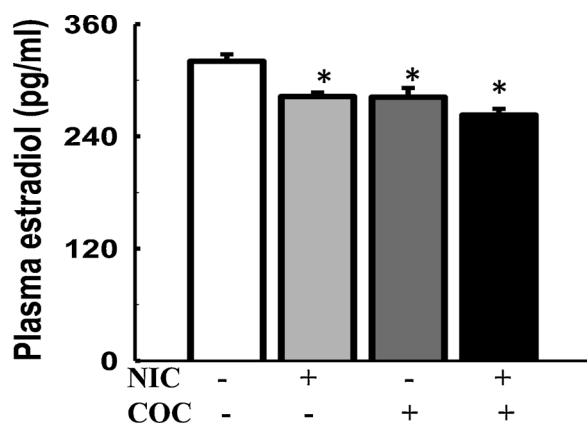


Fig. 7. COC and NIC treatment decreased circulating oestradiol level in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * $p < 0.05$ vs CON, # $p < 0.05$ vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive.

level is negatively correlated with the incidence of the cardiometabolic disorder and inflammatory cytokines. It might be used as a predictor for the cardiometabolic disorder [41]. However, the reduced cardiorenal GSH, bilirubin levels and increased MDA level were reversed by COC + NIC treatment. This implicates the antioxidant potential of combined estrogen-progestin oral contraceptive and nicotine treatment.

Furthermore, NIC exposure or COC + NIC treatment resulted in increased plasma and renal cotinine levels whereas cotinine was not detected in the heart of COC + NIC-treated rats. As expected, cotinine was not detected in rats that were not exposed to nicotine. Cotinine, a major nicotine metabolite used as a marker of nicotine exposure, has been reported to have a considerably longer half-life than nicotine, between 19 and 24 h [42]. However, the finding of the present study revealed that cotinine level was more in NIC-exposed rats implying that cotinine may be in part responsible for the NIC-mediated cardiometabolic activities in these animals. In addition, COC + NIC treatment led to reduced cotinine levels compared to NIC-exposed groups. This is in line with the report that oral contraceptive increases nicotine metabolism and clearance [22]. This means that cotinine accumulates more in the NIC-exposed rats than in COC + NIC-treated group.

Furthermore, in line with our finding of reduced Na^+/K^+ -ATPase

activity in COC-treated rats, repressed Na^+/K^+ -ATPase enzyme activity has been associated with IR, hyperinsulinemia, hyperglycemia, diabetes, dyslipidemia, obesity and metabolic syndrome [43]. Similarly, in consonance with the findings of the present study, cholesterol accumulation has been reported to inhibit Na^+/K^+ -ATPase activity in the membranes of human erythrocytes, rat liver cells and rabbit kidney outer medulla cells by increasing membrane rigidity [44,45]. However, despite normoglycemia, COC treatment in the current study caused IR and hyperinsulinemia which was accompanied by suppressed cardiac Na^+/K^+ -ATPase activity. This is in agreement with the study that associated decreased cardiac Na^+/K^+ -ATPase activity with deficiencies in cardiac contractility in animal models and heart failure patients [46]. However, NIC-exposed rats with cardiometabolic dysregulation did not have any alteration in cardiac Na^+/K^+ -ATPase activity whereas renal Na^+/K^+ -ATPase activity was not affected by the treatment. The present finding that NIC exposure did not alter cardiac and renal Na^+/K^+ -ATPase activities is not in agreement with studies that associated chronic NIC exposure with decreased Na^+/K^+ -ATPase activity in skeletal muscle and brain of rats [47]. Interestingly COC + NIC treatment led to enhanced cardiac and preserved renal Na^+/K^+ -ATPase activities. The ameliorative effects of COC + NIC treatment on NIC or COC-induced cardiometabolic dysfunction and impaired cardiorenal Na^+/K^+ -ATPase activities is supported by reports that both anti-diabetic (e.g. metformin and statins) and antihypertensive (losartan and enalapril) agents cause increased Na^+/K^+ -ATPase activity of erythrocytes in diabetic, dyslipidemic patients [48,49] and in the failing heart of rats after myocardial infarction [50] respectively.

More so, NIC or COC-induced cardiorenal dysfunction is associated with decreased circulating estradiol in the present study. This supports the reports that ovariectomized rats exhibited a decreased Na^+/K^+ -ATPase activity [51,52]. In contrast, increased estradiol has been reported to enhance Na^+/K^+ -ATPase activity in human and rat cardiomyocytes [53]. Besides, a study also reported increased erythrocytes Na^+/K^+ -ATPase activity in women when their estradiol levels reached its peak during the menstrual cycle [54]. Interestingly, the finding that COC + NIC treatment increases cardiac Na^+/K^+ -ATPase activity and preserved renal Na^+/K^+ -ATPase activity regardless of decreased circulating estradiol level in COC + NIC-treated rats is suggestive of the fact that the cardiorenal beneficial effects of COC + NIC treatment may not be dependent on the circulating estrogen levels. However, this may not be reflective of the intracellular (local) estrogen concentration especially in the cardiac and renal tissues. This is supported by the

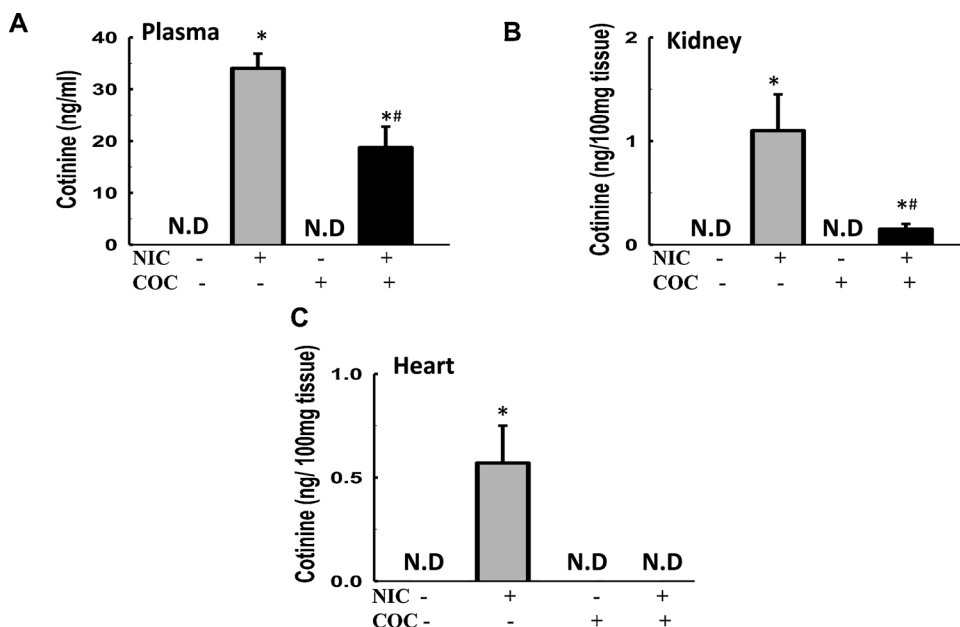


Fig. 8. COC and NIC treatment increased circulating and renal cotinine (A & B) but cardiac cotinine (C) level was undetectable in female Wistar rats. Values are expressed as mean $\pm$ SEM of 6 rats per group; * $p < 0.05$ vs CON, # $p < 0.05$ vs COC group. Data were analyzed by one-way ANOVA and Bonferroni's Post hoc analysis; CON; control, NIC; nicotine, COC; combined oral contraceptive.

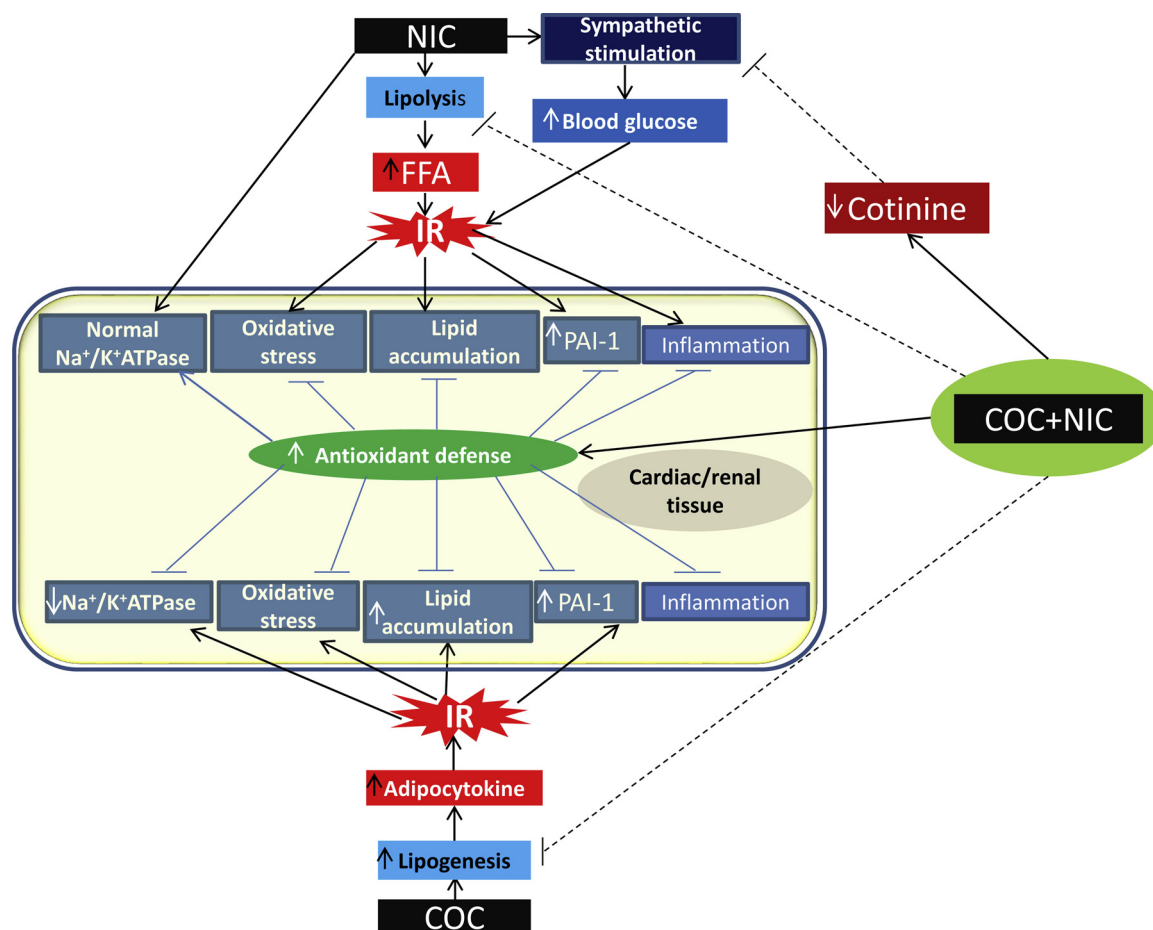


Fig. 9. Schematic diagram illustrating the likely pathway through which COC + NIC treatment synergistically confer cardio-renal protection. COC: combined oral contraceptive, NIC: nicotine, IR: insulin resistance, FFA: free fatty acid, PAI-1: plasminogen activator inhibitor-1.

report that estrogen synthesis and signaling is both tissue and cell specific. This means that irrespective of low circulating levels, local estrogen concentrations may be high, reflecting on their biological actions [55]. Unlike ovarian-synthesized estrogen, which is mainly released into the bloodstream, estrogen synthesized within extragonadal structures (such as kidney, heart, adipose tissue, skin, and brain) mostly acts locally at the site of synthesis and functions as a paracrine and/or intracrine factor to preserve essential tissue specific functions [56,57]. Therefore, COC + NIC treatment may provide therapeutic potential for atherothrombotic CVD events and renal dysfunctions in NIC-exposed or COC-treated rats by possibly modulating intracellular estrogen signaling and synthesis.

Furthermore, nicotine is known to be a potent aromatase inhibitor which is responsible for the observed anti-estrogenic action in the present study [58]. Aromatase inhibition prevents the conversion of androgen to estrogen resulting in elevated circulating androgen especially the free testosterone and this might be responsible for the deleterious actions of nicotine in the female rats. This may create a metabolic dysregulation similar to what is observed in polycystic ovarian syndrome (PCOS). Interestingly estrogen-progestin COC is also known to possess anti-androgen action [59]. In particular, circulating testosterone, the most potent circulating androgen in women, was reported to decrease by up to 50% [60]. This is why COCs are the most often used treatment modality for PCOS [61]. The three probable crucial mechanisms responsible for COC-mediated anti-androgenic effect are; suppression of ovarian androgen synthesis, increased sex hormone binding globulin (SHBG) levels, and suppression of adrenal androgen synthesis [59]. Increase SHBG results in decrease free testosterone levels twice as much as total testosterone. The estrogen dose and

progestin type of the COC do not influence the decline of total and free testosterone [62]. This interaction between COCs, nicotine and sex hormones (androgen and estrogen) may play a large part in the observed cardiorenal metabolic protective effect of combined estrogen-progestin therapy and nicotine exposure in the present study.

Growing evidence demonstrates that hyperuricemia is an independent risk factor for cardiovascular and renal diseases [18,63]. The finding that NIC exposure or COC treatment led to elevated cardiorenal uric acid level is suggestive of potential cardiorenal damage because cardiac and renal uric acid accumulation has been associated with heart failure and chronic kidney disease respectively. In line with this finding, evidence suggests that uric acid may either be a biomarker of poor prognosis or an active player in the pathogenesis of heart failure [64,65]. Furthermore, studies have reported that cardiac and renal remodelling accompanying cardiometabolic disorder is often characterized by increased cardiorenal fibrosis, inflammation and stiffness [66]. This is also in agreement with the findings of the current study that NIC exposure or COC treatment led to elevated cardiorenal PAI-1 (prothrombotic marker) and uric acid (inflammatory marker) levels. In patients with heart failure, hyperuricemia has been shown to be associated with inflammatory-mediated endothelial damage [67]. Therefore, nicotine or COC treatment may mediate its deleterious cardiorenal effects through uric acid/oxidative stress/inflammation-dependent pathway. However, the finding that COC + NIC treatment led to a reduction in cardiac and renal uric acid level is fascinating and may be responsible for the amelioration of cardiometabolic disturbances observed in this group of animals. Hence, the uric acid lowering properties of COC + NIC treatment may be a pharmacological target for the management of the inflammation and/or oxidative stress-mediated

metabolic disturbances and cardiorenal dysfunctions associated with either COC or nicotine singly administration.

Unraveling the mechanisms responsible for inflammation and its effects on tissues is vital to the understanding of many pathological conditions. Hence, the report that glycogen synthase kinase-3 (GSK-3) promotes inflammation unlocks a new component of its well documented actions in several prevalent diseases that involve inflammation, including diabetes, metabolic syndrome, and cancer [68]. The finding in the present study that NIC exposure or COC treatment led to elevated cardiorenal GSK-3 concentration and increased inflammatory responses as highlighted by the elevated pro-inflammatory biomarker (uric acid) in this study signify a connection between GSK-3 and inflammation. Therefore, NIC or COC-induced inflammatory responses could be due to a GSK-3 and/or uric acid-dependent mechanism. Surprisingly, COC + NIC treatment result in reduced cardiac and renal GSK-3 concentration which coincides with the observed anti-inflammatory effects of COC + NIC treatment as previously reported [32]. This raises the possibility that COC + NIC treatment may be an inhibitor of GSK-3 which can be beneficial in many inflammation-related conditions. GSK-3 inhibitors have been reported to reduce systemic inflammatory response and renal dysfunction [69]. Therefore, GSK-3 plays a significant role in inflammation and COC + NIC treatment could be a potential GSK-3 inhibitor mediating its anti-inflammatory effects through a GSK-3 inhibitory mechanism. Taken together, estrogen-progestin oral contraceptive and nicotine exposure counteracts COC or NIC-induced cardiometabolic disorder with associated cardiorenal dysfunction is accompanied with decreased cardiorenal uric acid and GSK-3 concentration.

5. Conclusion

The present study demonstrates that oral estrogen-progestin therapy and nicotine exposure interact to prevent IR-linked cardio-renal toxicity by attenuating cardiac and renal lipid accumulation, oxidative stress, inflammation and impaired Na^+/K^+ -ATPase activity, which is mediated by uric acid/GSK-3-dependent mechanism. Therefore, the use of estrogen-progestin oral contraceptive during nicotine exposure synergistically preserves cardiorenal metabolic function via uric acid/GSK-3-dependent mechanism.

Funding sources

This research was done without specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Authors' contributions

OLA conceived and designed the research. MOS and AED conducted experiments. MOS and OLA contributed new reagents and analytical kits. MOS, AOA, OKS and AED analyzed and interpreted data. MOS, DCL, AOA and OKS drafted the manuscript. All authors read and approved the manuscript for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors appreciate the technical support from the HOPE Cardiometabolic Research Team, Ilorin, Nigeria.

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