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ORIGINAL ARTICLE



Allopurinol and valproic acid improve cardiac triglyceride and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity independent of circulating aldosterone in female rats with glucose intolerance

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ABSTRACT

Context: Studies have shown that cardiac triglyceride accumulation and impaired $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity are linked to diabetes-related cardiovascular disease, particularly in women.

Objectives: We hypothesised that allopurinol (ALL) and valproic acid (VPA) treatment would improve cardiac triglyceride and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity independent of circulating aldosterone in Combined Oral Contraceptive (COC)-induced dysglycemia

Materials and methods: Rats received COC (1.0 μg ethinylestradiol and 5.0 μg levonorgestrel; *po*) with or without ALL (1 mg; *po*) and VPA (20 mg; *po*) for 6 weeks.

Results: COC-treatment led to impaired glucose tolerance, accumulated abdominal fat, dyslipidemia, elevated plasma MDA, PAI-1 and aldosterone levels and also reduced plasma nitric oxide bioavailability and cardiac $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. However, either ALL or VPA treatment ameliorated these alterations comparably independent of elevated aldosterone level

Discussion and conclusion: Our results suggest that either ALL or VPA would improve cardiac TG and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity comparably in COC-treated rats, regardless of circulating aldosterone level.

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Introduction

Cardiovascular disease (CVD) represents the major cause of mortality in both developed and developing countries (GBD 2015). Women are at greater risk of metabolic syndrome-associated CVD particularly after menopause (Eshtiaqui *et al.* 2010). However studies including research from our laboratory have demonstrated that continuous usage of estrogen-progestin formulations of oral contraceptives (COC) is associated with insulin resistance in premenopausal women (Jiang *et al.* 2014) and young female rodents (Olatunji *et al.* 2016). Impaired glucose tolerance, hyperinsulinemia, dyslipidemia with elevated triglyceride level and endothelial dysfunction are well-documented risk factors for cardiovascular disease (CVD) (Calles-Escandon and Cipolla 2001, Gast *et al.* 2012). Despite massive existing findings on cardiometabolic issues in women, there is an increasing need to fully elucidate therapeutic targets for new or existing drugs to effectively manage cardiometabolic disease and reduce CVD-related deaths particularly in women taking COC.

Visceral obesity is an independent risk factor of cardiometabolic disease that adversely impacts cardiac structure and function. Generally, the IR state is characterised by an expanded visceral adipose tissue beyond fat storage capacity resulting in the overflow of FFAs into circulation from where depositions occur in metabolic tissues particularly the heart where fat build up occur in form of triglyceride (Fox *et al.* 2007, Yazıcı and Sezer 2017).

Plasminogen activator inhibitor (PAI-1), a member of the serine protease inhibitor (serpin) family is an inhibitor of the fibrinolytic system that counteracts the effect of plasminogen activators, thereby inhibiting plasmin formation and down-regulating breakdown of fibrin clots resulting in hypercoagulation of blood (Cesari *et al.* 2010). Raised plasma PAI-1 levels have been identified as a predictor of atherothrombosis and myocardial infarction (Juhan-Vague *et al.* 1996, Tjärnlund-Wolf *et al.* 2012). Studies have shown the exclusive association between high plasma PAI-1 levels and visceral obesity and type 2 diabetes mellitus (Cigolini *et al.* 1996, Lyon and Hsueh 2003, Aso 2006).

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Na⁺/K⁺-ATPase (NPA) is the primary Na⁺ extrusion pathway in cardiac myocytes, consuming ATP to pump 3 Na⁺ ions out, in exchange for 2 K⁺ ions, which determines the driving force for Na⁺ entry into the myocyte. Present in all mammalian cells, NPA maintains the electrochemical gradient of Na⁺ and modulates cardiac contractility by influencing the Na⁺/Ca²⁺ exchange. It also plays a central role in the regulation of cell ion content, and cell volume (Lingrel and Kuntzweiler 1994, Hilgemann *et al.* 2006). Impaired NPA activity has been implicated in insulin resistance, glucose intolerance and atherosclerosis (Jorgensen *et al.* 2003). Also, NPA expression and activity are impaired in cardiac muscle from patients with coronary heart disease (CHD) (Müller-Ehmsen *et al.* 2002). CHD is highly prevalent among individuals with IR/diabetes and is the leading cause of death in both sexes globally. IR predisposes individuals to ischaemic cardiomyopathy, and contributes to an increased risk of heart failure (Mehta *et al.* 2015). Interestingly, women with IR or diabetes develop clinically significant atherothrombotic cardiovascular disease (CVD), particularly CHD not only readily or more frequently but also more severely than men with IR or diabetes. Hence, the disproportionate occurrence of atherothrombotic CVD, in women with IR.

Na⁺ influx and efflux play an important role in the pathogenesis of heart disease, as these systems are responsible not only for initiating the cardiac action potential (via voltage-gated Na⁺ channels) but also closely regulate the influx and efflux of Ca²⁺ through the Na⁺/Ca²⁺ exchanger (NCX1 in the heart) (Bers and Despa 2006).

Multiple studies have indicated that intracellular Na⁺ concentration ([Na⁺]_i) is increased in several animal models of cardiac diseases, including cardiac hypertrophy (Pogwizd 2003), as well as in human heart failure (Pieske and Houser 2003). This increased [Na⁺]_i in cardiac disease is likely an adaptive mechanism to reduce Ca²⁺ extrusion via NCX1 to augment contractility and cardiac function. However, this increase in cytosolic Ca²⁺ also increases the propensity for arrhythmias and may activate Ca²⁺ dependent signalling pathways involved in the hypertrophic programme and/or apoptosis (Goonasekera *et al.* 2012).

Kidneys are considered classical targets for aldosterone. When extracellular fluid volume is depleted, the renin-angiotensin-aldosterone system becomes activated, leading to increased release of aldosterone. The increase in circulating aldosterone stimulates renal Na⁺ retention. Furthermore, aldosterone can bind with high affinity in the heart (Pearce and Funder 1987). Its effects on the cardiac Na⁺/K⁺ pump have been examined with several reports on its variable effects (Robert *et al.* 1999, Bueno-Orovio *et al.* 2014).

Repositioning of drugs targeted at decreasing the risk of cardiovascular complications in IR/diabetic patients have in recent times been of utmost interest. Allopurinol, a xanthine oxidase inhibitor, has been shown to attenuate endothelial dysfunction, inflammation and progression of atherosclerosis by inhibition of uric acid production in humans and experimental models (Levy *et al.* 2018). Notwithstanding, the use of allopurinol in treatment of CVD is unresolved, particularly in patients with normouricemia (Gingles *et al.* 2019, Zhang

et al. 2019). Valproic acid (VPA) is a short chain fatty with well known anti-epileptic effect. Interestingly, reports from several studies showed that VPA may inhibit histone deacetylase thereby exerting ameliorative benefits in cardiometabolic disorders (Zhu *et al.* 2004, McKinsey 2011). In our recent study, VPA significantly suppressed hepatic damage by decreasing triglyceride accumulation and uric acid induced oxidative stress (Omolekulo *et al.* 2018). It was also observed in the course of this current study that allopurinol and valproic acid attenuates COC-induced increase in circulating uric acid (see [supplementary data](#)). There is a dearth of information regarding the effects of allopurinol and valproic acid treatment for CVD, especially their role in modulating endothelial function and cardiac Na⁺/K⁺-ATPase activity in COC users. Therefore, the present study sought to address the role of PAI and cardiac Na⁺/K⁺-ATPase in COC-induced IR. In addition, this study hypothesised that the mechanism by which allopurinol and valproic acid improve cardiac dysfunction in COC treated female rats is via attenuation of circulating aldosterone level.

Materials and methods

Animals

The investigation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Ethical Review Board of University of Ilorin, and every effort was made to minimise both the number of animals used and their suffering. Ten weeks old female Wistar rats were obtained from the animal house of the College of Health Sciences, University of Ilorin (Ilorin, Nigeria). Rats had unrestricted access to standard rat chow and tap water. After one week of acclimatisation, the animals were randomly assigned to 6 groups ($n=6/\text{group}$). Rats were maintained under standard environmental conditions of temperature, relative humidity, and 12-h dark/light cycle.

Treatment

Control group received distilled water (vehicle; *po*) daily. COC-treated group received (*po*) COC; a combination of 1.0 µg ethinylestradiol and 5.0 µg levonorgestrel (Wyeth–Ayerst, Inc., Montreal, Canada) daily via 0.2 ml of vehicle based on previous studies (Olatunji *et al.* 2016). COC+ALL-treated group received (*po*) a combination of COC plus allopurinol (ALL; 5 mg/kg *bw*) daily (Aspen Bad Oldesloe) based on previous studies (Hu *et al.* 2009). ALL was administered via 0.3 ml of vehicle. COC+VPA-treated group received a combination of COC plus valproic acid (VPA; 100 mg/kg *bw*) (Sanofi-Synthelabo Limited, United Kingdom) daily. The treatment lasted for 6 weeks.

Sample preparation

At the end of treatment, the rats were anaesthetised with pentobarbital sodium (50 mg/kg, *ip*). Blood was collected by

cardiac puncture into heparinised bottle and was centrifuged at 3000 rpm for 5 min at room temperature. Plasma was stored frozen until needed for biochemical assay. The heart was excised, cleared of adhering connective tissues, blotted and weighed immediately. After weighing, 100 mg of heart tissue was carefully removed and homogenised with a glass homogeniser in cold phosphate-buffered

Glucose tolerance

Glucose challenge test was performed 48 h before the end of the experiment. The rats had a 12-h overnight fast. Glucose (2 g/kg *bw*) was given (*po*). A tail capillary droplet of blood was used to assess blood glucose sequentially after 30, 60, 90 and 120 min using a glucometer (ONETOUCH®-LifeScan, Inc., Milpitas, CA, USA). Glucose tolerance was expressed as a function of the area under the curve (AUC) of oral glucose tolerance test (OGTT) and postload glucose responses were estimated. Elevated 1-h postload glucose level is also used as a reliable predictor of IR, pancreatic β -cell function, atherosclerotic CVD (Succurro *et al.* 2010, Marini *et al.* 2013).

Biochemical assays

Plasma levels of PAI-1 and aldosterone were determined using ELISA kits obtained from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Plasma levels of triglyceride (TG) was measured by standardised colorimetric method using assay kits obtained from Fortress Diagnostics Ltd. (Antrim, UK). Plasma nitric oxide was measured by quantification of nitrite using Griess Reagent and malondialdehyde (MDA), as marker of oxidative stress was measured by standardised enzymatic colorimetric methods using assay kits from Oxford Biomedical Research, Inc. (Oxford, MI, USA).

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 $p < .05$.

Results

Allopurinol and valproic acid decrease visceral fat mass, have no effects on body and cardiac weights whereas only valproic acid improves glucose tolerance in COC-treated rats

Body and cardiac weights were not affected in all experimental groups (Figure 1(a,b,d)). COC treatment led to a significant increase in visceral fat mass when compared to control. However, treatment with COC + ALL and COC + VPA reduced visceral fat mass when compared to COC-treated rats (Figure 1(c)). COC treatment with or without ALL significantly

increased AUC level compared to control. Nevertheless, AUC value was attenuated in COC + VPA-treated rats when compared with COC-treated rats (Figure 1(e)).

Allopurinol and valproic acid reduce cardiac triglyceride (TG) accumulation and lipid peroxidation in COC-treated rats

COC-treatment led to cardiac TG accumulation when compared to control. However, treatment with COC + ALL and COC + VPA reduced cardiac TG accumulation when compared with COC-treated group (Figure 2(a)). Cardiac malondialdehyde (MDA) level, a marker of lipid peroxidation was significantly increased in COC-treated rats when compared with control. Nevertheless, ALL and VPA treatment significantly reduced cardiac MDA level in COC-treated rats (Figure 2(b)).

Allopurinol and valproic acid increase plasma nitric oxide level and cardiac $\text{Na}^+\text{-K}^+$ -ATPase activity but decreased plasma plasminogen activator-inhibitor-1 (PAI-1) level in COC-treated rats

COC-treatment led to a significant increase in plasma PAI-1 level when compared to the controls (Figure 3(a)). However, COC + ALL and COC + VPA treatment led to a significant reduction in plasma PAI-1 level when compared to COC-treated rats (Figure 3(a)). On the other hand, there was a significant decrease in the circulating nitric oxide level in COC-treated rats when compared to control whereas COC + ALL and COC + VPA significantly increased circulating nitric oxide level compared to COC-treated rats (Figure 3(b)). COC treatment also led to decrease in cardiac $\text{Na}^+\text{-K}^+$ -ATPase activity when compared with the control group (Figure 3(c)). This decrease was however reversed in the COC + ALL and/or COC + VPA groups when compared with the COC only group.

Allopurinol and valproic acid increase aldosterone in COC-treated rats

Plasma aldosterone level was elevated comparably in all treated groups when compared to control group (Figure 4).

Discussion

The main findings from our current study reveals that treatment of female rats with combined OC steroids induced cardiometabolic syndrome as evident by impaired glucose tolerance, cardiac triglyceride accumulation and lipid peroxidation marker. This model of cardiometabolic syndrome is also accompanied by endothelial dysfunction mediators. Impaired glucoregulation is a critical feature in IR-related disturbances that include diabetes mellitus, atherothrombotic infarction and hypertension (Balkau *et al.* 1993, Wei *et al.* 1998, Tun *et al.* 2017).

Similar to our previous study on OC steroids in this study did not affect cardiac weight (Olatunji *et al.* 2017). Also, there

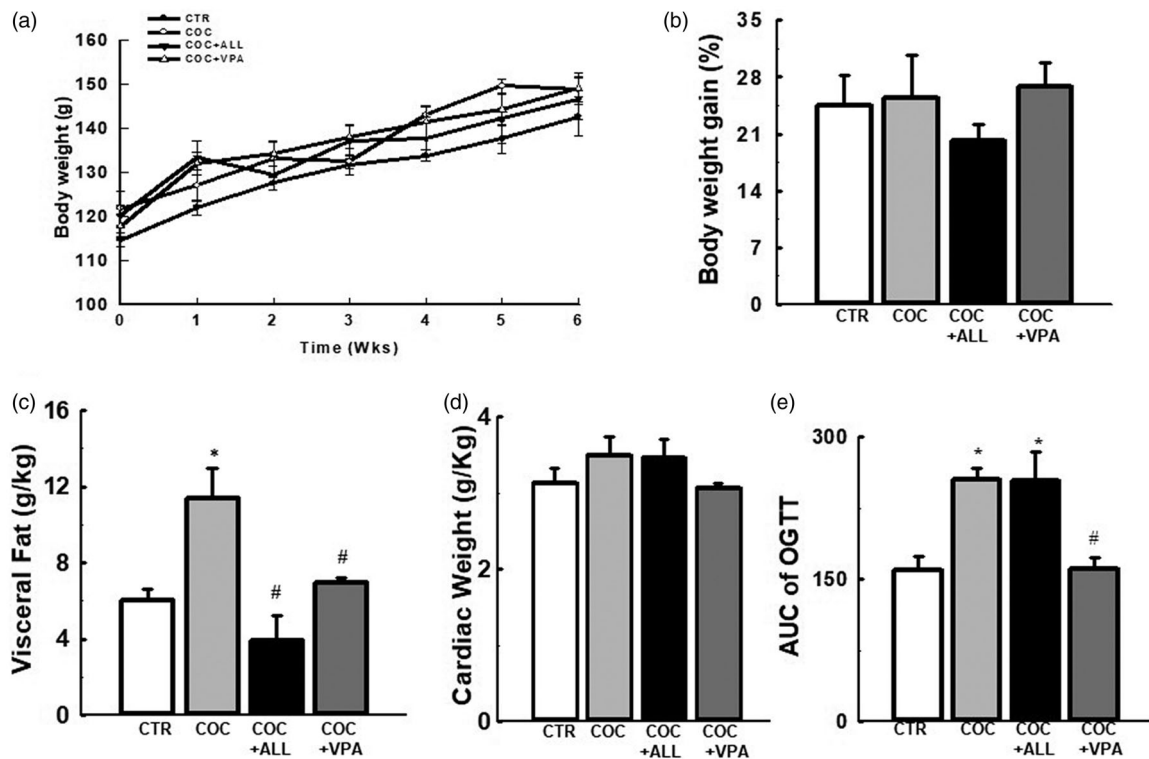


Figure 1. Effect of allopurinol and valproic acid on total body weight (a), body weight gain (b), visceral fat mass (c), cardiac weight (d), and area under curve (AUC) of oral glucose tolerance (OGTT; e) in COC-treated rats. Allopurinol and valproic acid led to a decrease in visceral fat mass but had no effects on body and cardiac weights. However, only valproic acid improves glucose tolerance in COC-treated rats. Values are presented as mean $\pm$ standard error of mean and $p < .05$ was taken as statistically significant. * $p < .05$ vs. Ctr; # $p < .05$ vs. COC. Data were analysed by one way ANOVA and Bonferroni's *Post hoc* analysis. CTRL: control; COC: combined oral contraceptive; ALL: allopurinol; VPA: valproic acid.

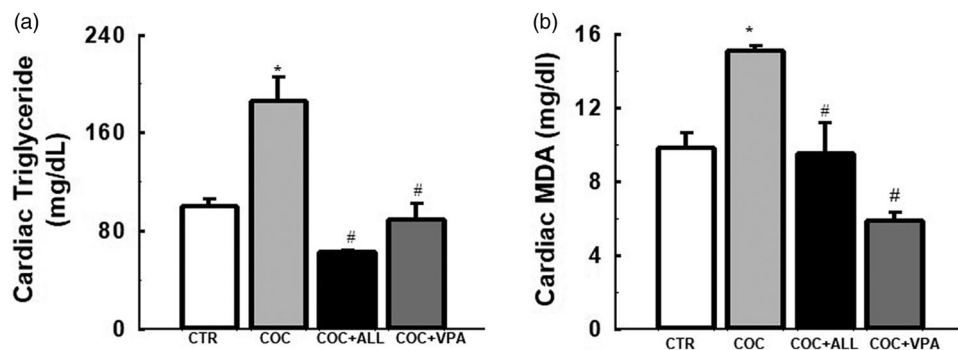


Figure 2. Effect of allopurinol and valproic acid on cardiac triglyceride (a) and malondialdehyde (MDA; b) in COC-treated rats. Allopurinol and valproic acid led to a reduction in cardiac triglyceride accumulation and lipid peroxidation in COC-treated rats. Values are presented as mean $\pm$ standard error of mean and $p < .05$ was taken as statistically significant. * $p < .05$ vs. Ctr; # $p < .05$ vs. COC. Data were analysed by one-way ANOVA and Bonferroni's *Post hoc* analysis. CTR: control; COC: combined oral contraceptive; ALL: allopurinol; VPA: valproic acid.

was no significant differences in body weight gain across the groups. However, a significant increase in visceral fat mass was observed when compared to control. Visceral obesity irrespective of body weight status is a phenotype that underlies IR and associated diseases (Dong *et al.* 2017). In this study, COC-treatment led to an increase in cardiac triacylglycerol level. Lipid accumulation in the heart has been implicated in human and animal models of cardiac hypertrophy. In IR state, expansion of visceral adipose tissue beyond its storage capacity causes an overflow of FFAs into circulation from where depositions occur in metabolic tissues particularly the heart (Yazıcı and Sezer 2017). Although FFAs is the major substrate for oxidative phosphorylation and ATP generation in cardiomyocytes, excess cardiac TG build up may

pose a lipotoxic effect. Furthermore, there was an increase in plasma cardiac malondialdehyde (MDA) level in the COC-treated rats compared to control, an index of oxidative stress and/or lipid peroxidation.

Data from the present study also revealed that glucose and lipid dysmetabolism is associated with elevated circulating PAI and NO, showing altered vascular homeostasis. PAI is an inhibitor of fibrinolysis thereby promoting arterothrombosis, stroke and coronary artery disease particularly in viscero-obese and type 2 diabetic patients. Reduced NO and raised circulating PAI in this study could be due to oxidative injury. Studies have shown that hyperglycemia-induced ROS production may directly scavenge NO or uncouple eNOS via oxidative modification of tetrahydrobiopterin (Landmesser

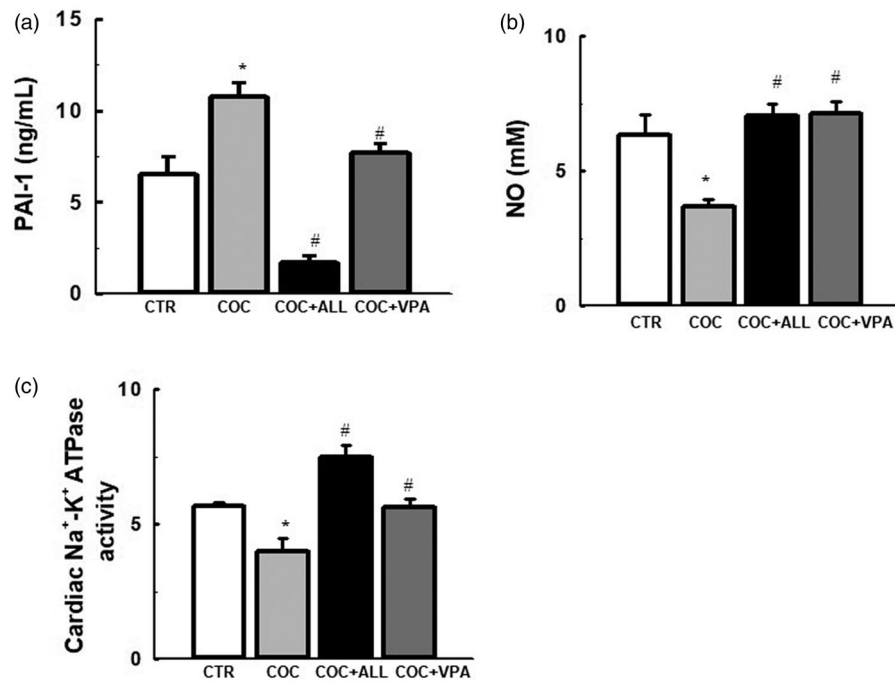


Figure 3. Effect of allopurinol and valproic acid on plasma plasminogen activator inhibitor-1 (PAI-1; a), nitric oxide (NO; b) and cardiac Na⁺-K⁺-ATPase activity (c) in COC-treated rats. Allopurinol and valproic acid led to an increase in plasma nitric oxide level and cardiac Na⁺-K⁺-ATPase activity when compared with the COC-treated group. However, only valproic acid caused a reduction in plasma plasminogen activator-inhibitor-1 (PAI-1) level when compared with the COC-treated group. Values are presented as mean $\pm$ standard error of mean and $p < .05$ was taken as statistically significant. * $p < .05$ vs. Ctr; # $p < .05$ vs. COC. Data were analysed by one-way ANOVA and Bonferroni's *post hoc* analysis. CTR: control; COC: combined oral contraceptive; ALL: allopurinol; VPA: valproic acid.

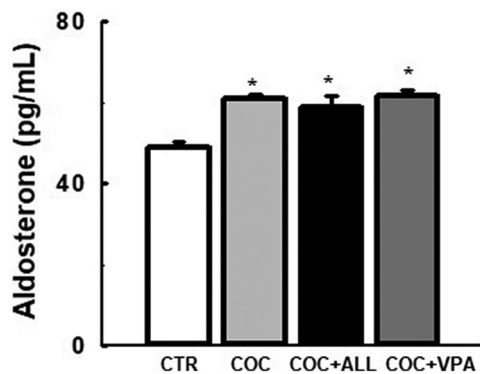


Figure 4. Effect of allopurinol and valproic acid on plasma aldosterone levels in COC-treated rats. There was a comparable increase in circulating aldosterone levels in all the treatment groups. Values are presented as mean $\pm$ standard error of mean and $p < .05$ was taken as statistically significant. * $p < .05$ vs. Ctr; # $p < .05$ vs. COC. Data were analysed by one-way ANOVA and Bonferroni's *post hoc* analysis. CTR: control; COC: combined oral contraceptive; ALL: allopurinol; VPA: valproic acid.

et al. 2003). Studies from our laboratory supported that endothelial dysfunction is associated with COC usage (Olatunji *et al.* 2017, Michael and Olatunji 2018). In addition, COC-treated rats had impaired Na/K-ATPase activity in the heart. NPA is an ATP-powered counter-transporter that maintains the electrochemical gradient of Na⁺ and modulates cardiac contractility by influencing the Na⁺/Ca²⁺ exchange hence, it plays crucial roles in ECF volume regulation and cardiac function. Our data shows that dysmetabolism alters cardiac NPA activity. A reduced cardiac Na/K-ATPase activity or expression was shown to contribute to impaired cardiac contractility in heart failure patients and rats (Schwinger 2003, Liu *et al.* 2016). The mechanism by which Na/K-ATPase

activity is impaired in metabolic disorder has not been fully elucidated. However, oxidative stress has been suggested to at least in part contribute to this condition. Increase ROS production can oxidise the Na/K-ATPase at its α/β subunits leading to suppression of its function. Our observation that inhibition of Na/K-ATPase activity is linked to oxidative stress is supported by similar findings in the myocardium of laboratory rats (Petrushanko *et al.* 2012).

Furthermore, our data showed that COC-treatment led to elevated plasma aldosterone level compared with control rats. This is in consonance with our previous study in COC induced cardiometabolic disorder (Michael and Olatunji 2018). Aldosterone plays a central role in the regulation of electrolyte and fluid volume and in blood pressure (BP) homeostasis via its interaction with mineralocorticoid receptor. Raised plasma aldosterone levels have been implicated in insulin resistance, dyslipidemia, diabetes and endothelial dysfunction (Pruthi *et al.* 2014, Kim *et al.* 2015).

Interestingly, treatments with allopurinol and Valproic acid attenuated dysmetabolism-driven endothelial and cardiac dysfunction (Figure 5). The ameliorative effect of allopurinol and Valproic acid in the present study is attributable to their efficacy in suppressing lipotoxicity-induced oxidative stress. Allopurinol is a known inhibitor of vascular inflammation and oxidative stress, while previous studies have shown that valproic acid, an antipsychotic drug can be re-programmed in management of metabolic syndrome (Levy *et al.* 2018, Omolekulo *et al.* 2018). In this study, treatments with allopurinol and valproic acid respectively had no effect on body and cardiac weight in the COC groups, however, there was significant decrease in visceral fat mass compared with controls. In addition, we observed that ALL significantly

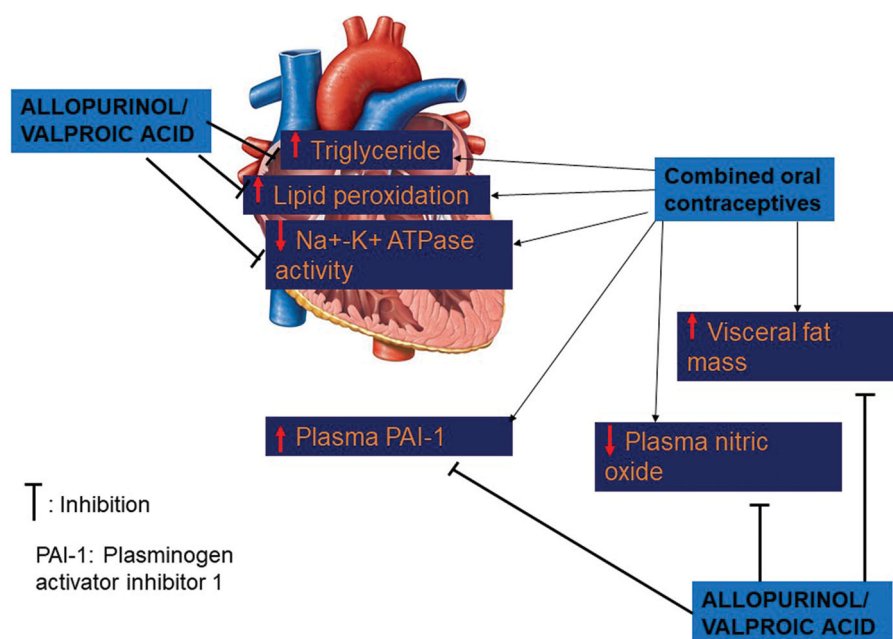


Figure 5. Graphical abstract showing the pathway of cardiac dysfunction after exposure to combined oral contraceptive and the ameliorative effect of allopurinol or valproic acid treatment on the cardiac impairments induced by combined oral contraceptives.

increased AUC level compared to control. Nevertheless, AUC value was attenuated in VPA-treated rats when compared with COC-treated rats. The mechanism by which ALL worsen glucose intolerance in COC-administered rats is unclear and may need subsequent studies for further explanations. Although a study reported that ALL did not reduce blood glucose level and insulin resistance in rat model of metabolic syndrome (El-Bassossy *et al.* 2015).

Also, cardiac TG and MDA levels were significantly attenuated in ALL- and VPA-treated groups versus COC groups. This finding is similar to our observation in COC-induced hepatic dysmetabolism in which VPA ameliorates oxidative injury through reduction of liver TG and MDA (Omolekulo *et al.* 2018). In addition, ALL- and VPA-treated groups had significant decrease in plasma levels of PAI and NO respectively. The mechanism by which ALL and VPA improve circulating levels of endothelial dysfunction markers in this study may be via the inhibition of lipotoxicity-induced oxidative stress.

Moreover, both ALL- and VPA-treatment significantly increase N/K-ATPase activity in COC administered rats, suggesting that either drugs could be used as effective therapeutic agents in reversing cardiac dysfunction. Several antihypertensives including antidiabetic drugs have been observed to enhance Na/K-ATPase activity in myocardial infarction rats. It is interesting to note that this study is the first to be reporting on the ameliorative benefit of ALL-treatment in COC-induced disrupted metabolism. Enhancement of Na/K-ATPase activity by ALL and VPA in this study is suggested to be via prevention of ROS oxidation of Na/K-ATPase by attenuation of cardiac TG accumulation and MDA. On the contrary, recent studies implicated ALL to impact some impairment in renal and left ventricular function (Gingles *et al.* 2019, Shen *et al.* 2019, Zhang *et al.* 2019). Data showed that ALL- and VPA- treatments did not alter aldosterone level

in COC rat group. This implicates that the ameliorative benefits of both drugs are not via the aldosterone mechanism.

In conclusion, the findings from this study show that lipotoxicity-induced oxidative stress and/or lipid peroxidation underlies disrupted vascular function and cardiac dysfunction in female rats given COC. Moreover, the mechanism by which ALL and VPA treatments improve COC impacts is independent of aldosterone but may be through prevention of ROS oxidation of Na/K-ATPase by suppressing cardiac TG accumulation and MDA. Hence, repositioning of either ALL and VPA to improve some components of cardiometabolic disorder, particularly plasma PAI-1 and cardiac Na/K-ATPase activity in glucose intolerant rats suggest that either drug could be an effective therapeutic option in the effort to reduce global morbidity or mortality from CVD.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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