



Endoglin inhibition by sodium acetate and flutamide ameliorates cardiac defective G6PD-dependent antioxidant defense in gestational testosterone-exposed rats



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ABSTRACT

Gestational androgen excess has been implicated in the development of cardiac dysfunction with poor mechanistic delineation. The role of sodium acetate on cardiac uric acid (UA) production and glucose-6-phosphate dehydrogenase (G6PD)-dependent antioxidant defense in pregnancy is not known. The study therefore sought to test the hypothesis that rats exposed to elevated testosterone in late pregnancy would have increased cardiac UA production and defective G6PD-dependent antioxidant defense. We also hypothesized that sodium acetate (SAC) or androgen receptor blocker, flutamide (Flu) would ameliorate these effects through endoglin inhibition. Twenty-four pregnant Wistar rats were treated (sc) with olive oil, testosterone propionate (0.5 mg/kg) singly or in combination with SAC (200 mg/kg; po) or Flu (7.5 mg/kg; po) in the late gestation between gestational day 14 and 19. The results showed that in the late gestation, testosterone exposure led to increased plasma and cardiac endoglin. In the heart of rats exposed to gestational testosterone there were elevated lactate dehydrogenase, adenosine deaminase, xanthine oxidase, uric acid (UA), cardiac injury markers and decreased G6PD-dependent antioxidant defense. However, either SAC or Flu comparably ameliorated these testosterone-induced effects. The data from the present study revealed that testosterone exposure in the late gestation causes elevated cardiac Eng that is accompanied by increased UA production and defective G6PD-dependent anti-oxidant defenses. Besides, the findings also suggest that the inhibitory effect of SAC or Flu on endoglin attenuates UA production and enhances the G6PD-dependent anti-oxidant barrier, thereby implying that endoglin may be a potentially novel therapeutic intervention for cardiac dysfunction particularly in pregnancy.

1. Introduction

Endoglin (Eng) is a 180-kDa transmembrane homodimeric glycoprotein, which is highly expressed in vascular endothelial cells as well as myocytes and hepatocytes [1,2]. Endoglin also functions as a non-signaling co-receptor of the transforming growth factor- β 1 (TGF- β 1) modulating its activities. Endoglin is a critical factor for angiogenesis, inflammation and wound healing, particularly in late pregnancy [3]. Recent studies have shown the positive association between endoglin and alterations in glucose metabolism. However, there is no information regarding the role of endoglin in cardiac adjustments in late pregnancy.

Adenosine regulates vascular inflammatory and cellular redox functions impacting protection on tissues against damage [4]. Also, its ability to mitigate renin-angiotensin system together with afore stated roles confer a protective shield on renal and cardiometabolic functions

[4–6]. Adenosine deaminase (ADA) is a polymorphic purine enzyme, which irreversibly catalyses conversion of adenosine to inosine. It is present in almost all mammalian cells and the activity has been reported to be prominent in lymphoid/immune cells and some metabolic tissues including liver and skeletal muscle. The activity of ADA has been correlated with inflammatory response through its ability to reduce adenosine [5,7]. Reduced serum activity of ADA has been observed in normal pregnancy compared to non-pregnant women whereas increased activity of ADA was observed in disorders of pregnancies such as preeclampsia [8]. Likewise, an increase in ADA activity was noted in non-pregnant T2DM patients [9]. Xanthine oxidase (XO) catalyzes the last two steps of purine metabolism modulating hypoxanthine to xanthine and then to uric acid [10]. Enhanced XO activity might be responsible for the pro-oxidant property of uric acid and its negative impact on cardiometabolic disorders [11]. XO uses molecular oxygen as an electron acceptor, thus generating superoxide and other reactive

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oxygen species (ROS) as by-product. This is responsible for the pathological conditions implicated in hyperuricemia [11]. Increase in uric acid production has been associated with several deleterious components of cardiovascular disease [12]. Recent findings also indicate that increased production of uric acid promotes cardiac hypertrophy, inflammation and oxidative stress, which in turn results in myocardial fibrosis and myocardial pathology [13].

Reactive oxygen species (ROS) promote or facilitate cardiac cell injury, playing critical roles in the pathophysiology of heart disease [14]. However, cardiomyocytes protect themselves against ROS via a highly active cellular antioxidant defense or endogenous thiol, reduced glutathione (GSH). GSH is capable of converting hydrogen peroxides and lipid peroxides to H₂O and lipid alcohols respectively [15]. GSH also plays an important role in protecting the oxidation of proteins sulfhydryl group. The maintenance of GSH pool ultimately requires G6PD, a rate limiting enzyme in the pentose phosphate pathway [15]. In non-nucleated mammalian cells, most importantly erythrocyte, G6PD is most established in the protection against cytosolic ROS [16,17]. Evidence also exists that decreased activity of G6PD is associated with diabetic or metabolic disorder-linked heart disease [18]. An increase in the activity of G6PD is generally considered to be protective against ROS by increasing the capacity of GSH defense system [19].

Short chain fatty acids (SCFAs) are end products from fermentation of complex carbohydrates and/or digestible fibres largely by colonic microbiota especially anaerobic bacteria [20]. Over 90% of the SCFAs present in the colon are constituted by acetate, propionate, and butyrate [21]. Acetate constitutes about 65% of the SCFA composition produced in the colon and it partially avoids hepatic clearance to reach micromolar concentrations in the systemic circulation [22]. SCFA treatment has been reported to ameliorate colitis, airway disease, kidney disease and metabolic syndrome in diet-induced obese mice [23]. Reports also showed that acetate decreased the lipopolysaccharide-induced production of intracellular reactive oxygen species (ROS) concomitant with an increase in glutathione [24]. Elevated testosterone in women with PCOS has been associated with altered gut microbiota and oxidative stress and is implicated in the pathogenesis of cardiac derangements [25,26]. However, despite the increased attention on SCFA in cardiometabolic disorders, there is lack of information in literature regarding the mechanism involved in the role of excess gestational androgen in cardiac oxidative stress-linked injury. We therefore tested the hypothesis that sodium acetate or blockade of androgen receptor would cause inhibition of endoglin and improve G6PD-dependent antioxidant capacity as well as reduce uric acid production with resultant attenuation of cardiac injury in the heart of animals exposed to late-gestational testosterone.

2. Materials and methods

2.1. Animals

The study was carried out in line with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was ratified by the ethical review committee, University of Ilorin, Ilorin, Nigeria. Number and suffering of animals were minimized. 130–150 g weight Pregnant Wistar rats obtained from the Animal House, College of Health Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria, were housed in a well-ventilated room kept under standard conditions of humidity, feeding, light and temperature. Unhampered access to standard rat chow and tap water were given to the rats. Twenty-four (24) rats were grouped into four (4) (n = 6/group) and were randomly assigned to vehicle (control; Ctr), testosterone-exposed (Tes), testosterone + flutamide (Tes + Flu) and testosterone + acetate (Tes + SAc) groups.

2.2. Treatment

The animals were treated with olive oil (sc) and with distilled water by gavage (Ctr), testosterone propionate in olive oil (0.5 mg/kg; sc) singly (Tes) or in combination with sodium acetate (Tes + SAc) in distilled water by gavage (200 mg/kg; po) or flutamide (Tes + Flu), an androgen receptor (AR) blocker in distilled water by gavage (7.5 mg/kg; po) from day 14 to 19 of gestation.

2.3. Sample preparation

2.3.1. Blood sample

At the termination of the experiment, 24 h prior to the end of the experiment, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) after an overnight 12-hourfast. Blood was collected by retro-orbital puncture into EDTA and heparinized bottles. After centrifugation of blood samples at 3000 rpm for 5 min, plasma was obtained and stored frozen until needed for biochemical assay.

2.3.2. Tissue sample

The hearts of the animals were excised and homogenized in phosphate buffer solution (PBS). The homogenized organs were centrifuged at 3000 rpm for 15 min and the homogenates were decanted and stored frozen until needed for biochemical assay.

2.3.3. Adipose tissue collection

Regional body fats were collected by opening up the rats' abdominal region after blood collection during the process of organ harvest. The fat pads collected were peri-renal, abdominal and peri-uterine fats. The fat pads were weighed and adjusted for body weight.

2.4. Biochemical assay

Plasma and cardiac endoglin were determined by using ELISA kit (E-EL-R2434) from Elabsience (Wuhan, China). Plasma and cardiac adenosine, adenosine deaminase (ADA), xanthine oxidase (XO), uric acid (UA), glucose-6-phosphate dehydrogenase (G6PD), oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione peroxidase (GPx), gamma glutamyltransferase (GGT), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured by standardized enzymatic colorimetric method using assay kit obtained from Fortress diagnostics (Antrim, United Kingdom) whereas cardiac malondialdehyde (MDA) was measured by non-enzymatic colorimetric assay obtained from Oxford Biomedical Research Inc. (USA). Reduced to oxidized glutathione ratio (GSH/GSSG) was used as an indicator of antioxidant capacity.

2.5. Statistical analysis

All data were expressed as means $\pm$ SEM. Statistical group analysis was performed with SPSS statistical software (Chicago, IL). 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 comparison of mean values among the groups. Statistically significant differences were accepted at $p < 0.05$.

3. Results

3.1. Effect of flutamide and acetate on body weight change, feed intake, visceral fat, and cardiac weight in late gestational testosterone exposure

Testosterone treatment led to decreased body weight gain (Fig. 1A) despite significant increase in food intake (Fig. 1D). Whereas, acetate and flutamide reversed the testosterone-induced reduction in body weight gain and increased food intake (Fig. 1D). Testosterone with or

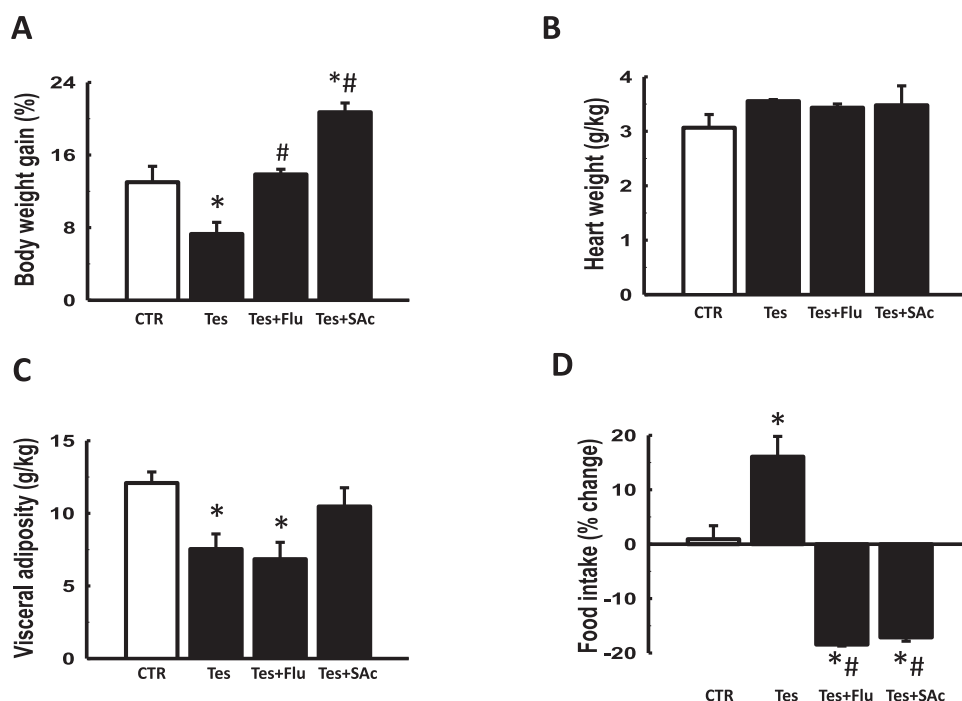


Fig. 1. Effect of flutamide (Flu) and sodium acetate (SAC) during late-gestational testosterone exposure on body weight gain (A) heart weight (B) visceral adiposity (C) food intake (D). Late-gestational testosterone led to decrease in body weight which was reversed by Flu and SAC. Late-gestational testosterone did not affect cardiac weight but reduced visceral adiposity which was not reversed by Flu and SAC. Late-gestational testosterone led to significant increase in food intake that was decreased by Flu and SAC. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values are expressed as mean ± SEM of 6 rats per group (* $p < 0.05$ vs control; # $p < 0.05$ vs Tes).

without AR blockade with flutamide but not acetate led to reduced regional body fat (Fig. 1C). Cardiac weights were comparable among experimental groups and maintained compared with the control (Fig. 1B).

3.2. Effect of flutamide and acetate on plasma and cardiac endoglin in late gestational testosterone exposure

Plasma and cardiac endoglin markedly increased with testosterone treatment compared with control (Fig. 2A and B), whereas testosterone with flutamide or acetate treatment attenuated the plasma and cardiac endoglin comparably (Fig. 2A and B).

3.3. Effect of flutamide and acetate on cardiac uric acid production and adenosine level in late-gestational testosterone exposure

Cardiac adenosine deaminase activity, xanthine oxidase activity and uric acid (uric acid production pathway) were increased with testosterone treatment compared with the control. These effects were attenuated by either acetate or flutamide (Fig. 3B–D). Adenosine level was reduced in all experimental groups compared with the control (Fig. 3A).

3.4. Effect of flutamide and acetate on cardiac lipid peroxidation index (MDA) and G6PD-dependent antioxidant capacity in late gestational testosterone exposure

Testosterone treatment led to increased cardiac MDA whereas, both AR blockade with flutamide and acetate attenuated MDA (Fig. 4A). Cardiac G6PD were comparable among experimental groups (Fig. 4B). However, reduced glutathione, glutathione peroxidase activity and antioxidant capacity index, GSH/GSSG were reduced with testosterone treatment and reversed by both flutamide and acetate treatment (Fig. 4C–D).

3.5. Effect of flutamide and acetate on cardiac injury prognostic enzymes and lactate dehydrogenase in late gestational testosterone exposure

Testosterone treatment resulted in increased cardiac GGT, ALT, AST and ALP. However, either AR blockade by flutamide or acetate treatment reversed the testosterone-induced increase in cardiac marker enzymes (Fig. 5A–D). Also, cardiac LDH indicating cell oxidative capacity increased with testosterone treatment, which was attenuated by either androgen receptor blockade by flutamide or acetate with testosterone treatment (Fig. 5E).

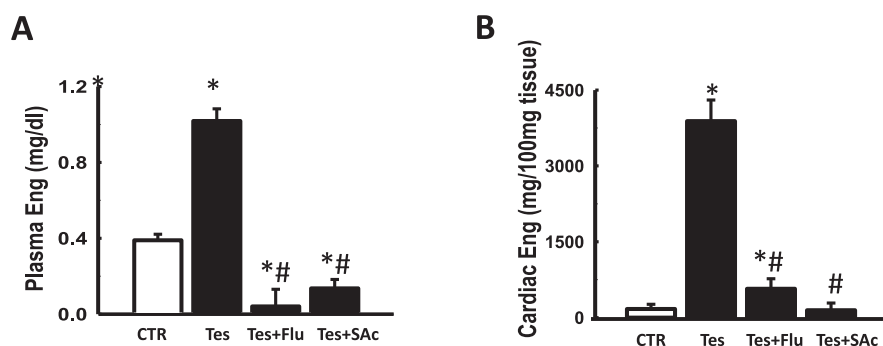


Fig. 2. Effect of flutamide (Flu) and sodium acetate (SAC) during late-gestational testosterone exposure on plasma endoglin (A) cardiac endoglin (B). Late-gestational testosterone led to increase in plasma and cardiac endoglin which were decreased by both Flu and SAC. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values are expressed as mean ± SEM of 6 rats per group (* $p < 0.05$ vs control; # $p < 0.05$ vs Tes).

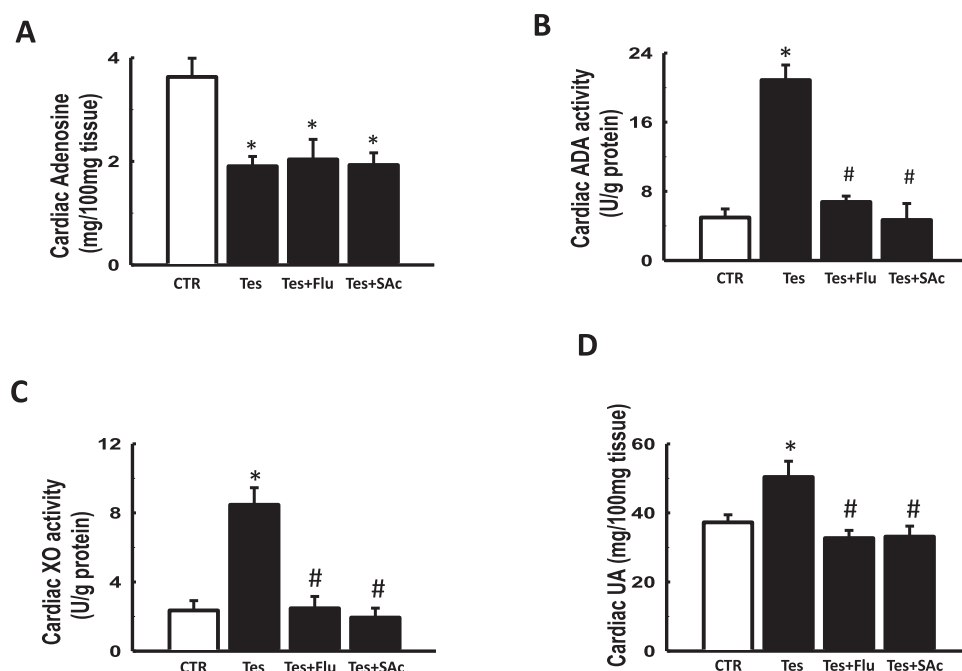


Fig. 3. Effect of flutamide (Flu) and sodium acetate (SAC) during late-gestational testosterone exposure on cardiac adenosine (A) cardiac adenosine deaminase (ADA) (B) cardiac xanthine oxidase (XO) (C) cardiac uric acid (UA) (D). Late-gestational testosterone exposure did not affect cardiac adenosine. Late-gestational testosterone exposure led to increased cardiac ADA, XO, UA which were attenuated by Flu and SAC. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* $p < 0.05$ vs control; # $p < 0.05$ vs Tes).

4. Discussion

Studies on female animals and humans unlike male have suggested that elevated androgen level especially during pregnancy is associated with deleterious cardiometabolic conditions [27], polycystic ovary syndrome (PCOS), hyperglycemia, gestational diabetes (GDM) and hypertension/preeclampsia [28] as well as poor fetal outcomes [29]. Oxidative stress, insulin resistance (IR) but not body weight phenotypes has been associated with testosterone-induced metabolic disturbances and cardiac dysfunctions [30–34]. However, the underlining mechanism of testosterone-induced oxidative stress is unresolved [34] and insights therein will assist to develop therapeutic measures for hyper-androgenism-linked (cardiac) disease/dysfunction in non-pregnant and pregnant women.

Regarding the involvement of XO in androgen-induced oxidative

stress, this study revealed in line with previous studies [31] that testosterone treatment at late gestation in rats resulted in increased cardiac activity of ADA/XO/UA pathway as well as lipid peroxidation, LDH and enzyme markers of tissue injury, which profoundly describe an oxidative status. Also, it is evident in this study that the oxidative status elicited by late-gestational testosterone exposure was strong enough to depress the G6PD-dependent antioxidant defense (Fig. 6). Interestingly, the outcome here is accompanied by several fold heightening of cardiac and plasma Eng with gestational testosterone treatment. However, findings here also show that androgen receptor (AR) blockade by flutamide in testosterone-exposed rats ameliorated cardiac oxidative stress and both cardiac and plasma Eng levels. Strikingly, treatment with acetate, a gut microbiota product also reproduced the AR blockade effect on testosterone-induced oxidative stress and Eng levels. Therefore, going by the outcome of this

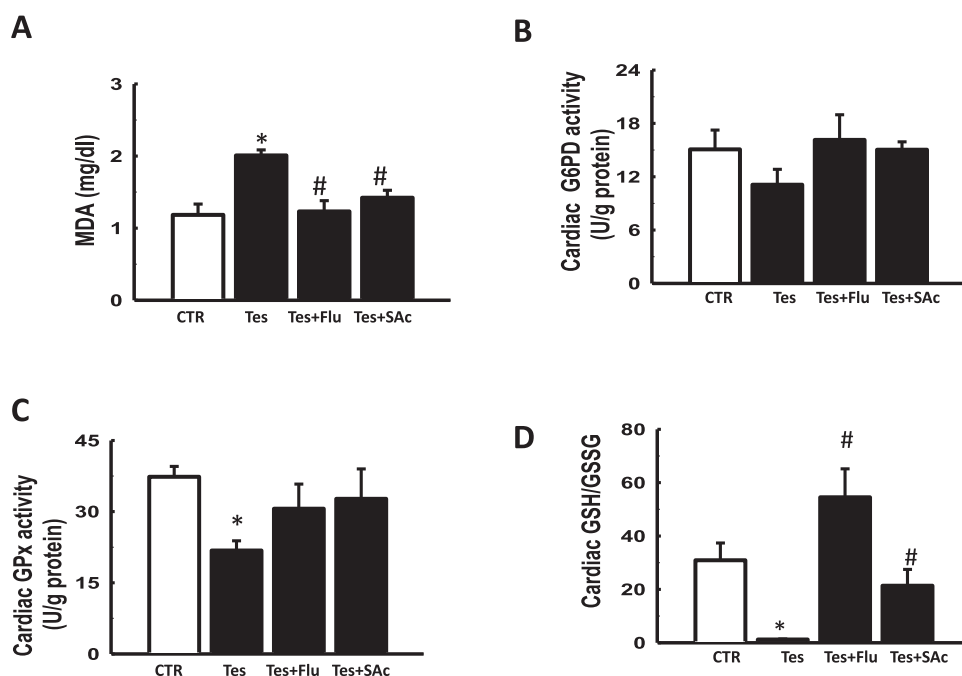


Fig. 4. Effect of flutamide (Flu) and sodium acetate (SAC) during late-gestational testosterone exposure on cardiac malondialdehyde (MDA) (A) cardiac glucose-6-phosphate dehydrogenase (G6PD) (B) cardiac glutathione peroxidase (GPx) (C) cardiac reduced: oxidized glutathione (GSH/GSSG) ratio (D). Late-gestational testosterone exposure led to increased cardiac MDA which was attenuated by Flu and SAC. Late-gestational testosterone exposure led to decreased G6PD and GPx which were not affected by Flu and SAC whereas decreased GSH/GSSG ratio was reversed by Flu and SAC. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* $p < 0.05$ vs control; # $p < 0.05$ vs Tes).

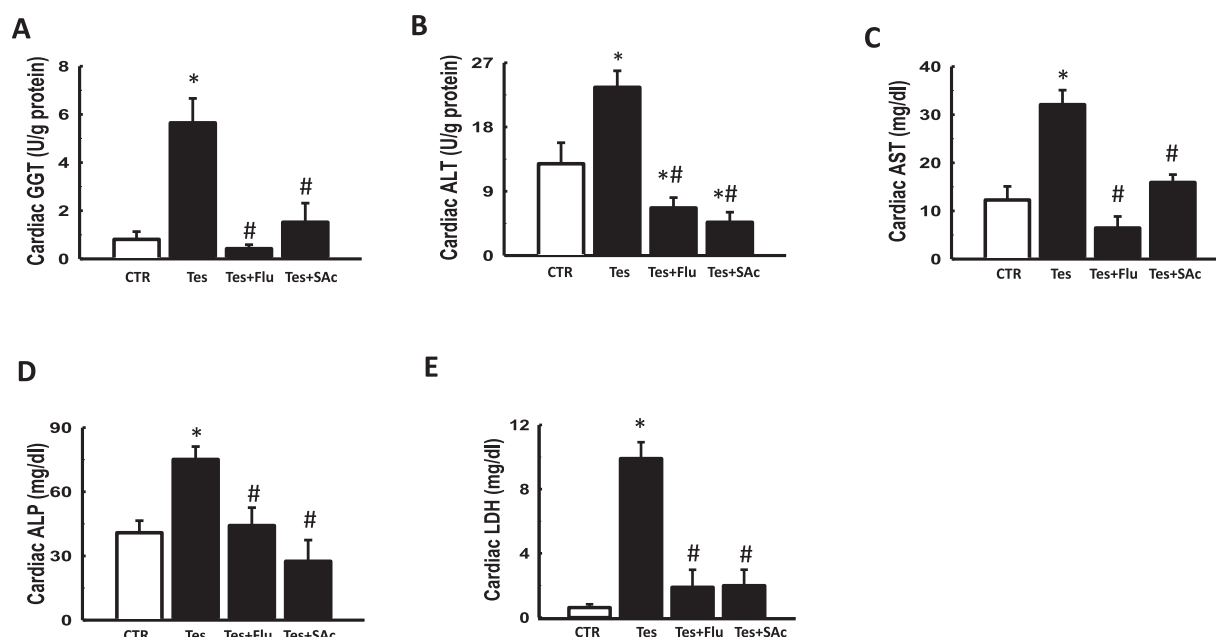


Fig. 5. Effect of flutamide (Flu) and sodium acetate (SAC) during late-gestational testosterone exposure on cardiac gamma-glutamyltransferase (GGT) (A) cardiac alanine transferase (ALT) (B) cardiac aspartate aminotransferase (AST) (C) cardiac alkaline phosphatase (ALP) (D) cardiac lactate dehydrogenase (LDH) (E). Late-gestational testosterone exposure led to increased cardiac GGT, ALT, AST, ALP and LDH and these were attenuated by both Flu and SAC. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. Values are expressed as mean $\pm$ SEM of 6 rats per group (* p < 0.05 vs control; # p < 0.05 vs Tes).

investigation, taking Eng as a novel therapeutic target for androgen-induced dysfunctions especially in females may be a milestone in the treatment of testosterone-engendered cardiometabolic disease in pregnancy and PCOS patients.

Supraphysiological levels of testosterone especially in females has been suggested to cause oxidative stress-mediated cardiovascular diseases [34]. Moreover, androgens has been shown to increase mitochondrial ROS and engender apoptosis in vascular smooth muscle cell (VSMC) [32]. The findings of this study agree with previous ones as stated above in showing the link between supraphysiological testosterone in females especially during pregnancy and tissue (cardiac) oxidative status. In this study, subcutaneous testosterone exposure to rats at late gestational period (14th–19th gestational days) increased cardiac lipid peroxidation/MDA level, enzyme markers of tissue injury

and depressed the G6PD-dependent glutathione antioxidant index/capacity. Lipid peroxidation is notorious for generation of lipid peroxides, which are oxidative species that along with others culminate damage to cellular structures, apoptosis and inflammatory/immune reactions in response to stress. Overwhelming oxidative stress will depress the antioxidant system and cause injury to tissues. In this study, G6PD, the cytosolic molecule that chiefly furnishes glutathione antioxidant system [19] was reduced along with GSH/GSSG index in testosterone-treated rats with accompanying elevated enzyme markers of tissue injury (Fig. 4). The most probable AR-dependent mechanisms of testosterone-engendered oxidative stress are the augmentation of mitochondrial ROS and enhanced NADPH oxidase, cyclooxygenase and xanthine oxidase activities among others as reported by a recent review (Tostes et al., 2016).

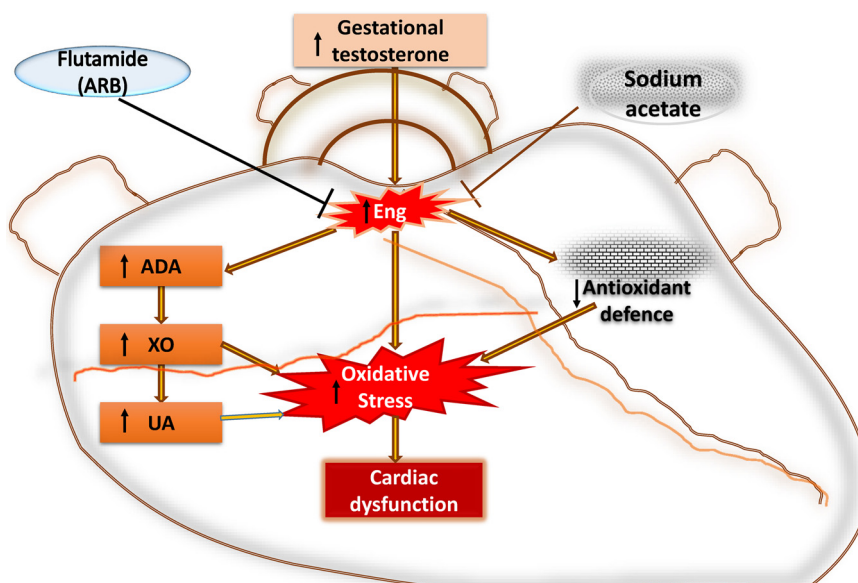


Fig. 6. Schematic diagram depicting the resolving role of flutamide and sodium acetate on cardiac dysfunctions caused by late-gestational testosterone exposure.

Conditions that overwhelm cellular antioxidant system fuels inflammatory response to stress and ADA has been implicated in immune-mediated inflammatory response [5]. In this study, testosterone treatment led to elevated cardiac ADA activity (Fig. 3b). ADA, degrades adenosine, an anti-inflammatory molecule commencing purine nucleoside degradation and signals the production of inflammatory cytokines from macrophages [7]. The pathway of purine degradation is another process associated with generation of oxidative species due primarily to the activity of XO in catalysing the last two steps of UA production [35]. Increased activity of this pathway has been associated with several metabolic disturbances and inhibition of XO has known cardiac therapeutic benefits [36]. In accordance with the previous findings on XO stated above, this study shows that testosterone treatment raised cardiac XO activity (Fig. 3c). This outcome is in line with the increased cardiac ADA activity with late gestational testosterone exposure since ADA supplies the substrate for XO activity. Besides, it gives evidence of oxidative stress-induced cardiac tissue injury caused by late gestational testosterone exposure. The enhanced activities of ADA and XO increases UA production. Increased uric acid production can inhibit production of nitric oxide by modulating arginase activity. Arginase is a negative regulator of L-arginine, the NO precursor. Reduced NO will compromise blood flow and lead to apoptotic and consequent fibrotic response.

This study establishes that elevated testosterone especially in late pregnancy can result in adverse oxidative status, which is implicated in IR and fibrotic response. The mechanism of testosterone-induced oxidative stress is poorly understood. However, the findings in this study on the effect of testosterone treatment in late gestation includes elevated cardiac and plasma Eng (Fig. 2). Eng is a coreceptor for TGF- β and is involved in the pathogenesis of preeclampsia [3] and fibrosis. There is evidence in this study by the several fold increase in cardiac Eng (Fig. 2a), that it is involved in testosterone-induced dysfunctions. Nevertheless, this study did not evaluate whether the testosterone-stimulated Eng expression is a genomic or non-genomic effect of AR activation and whether it is upstream or downstream of the oxidative stress.

AR blockade has known beneficial effect in androgen-induced endothelial dysfunction and insulin resistance in female dihydrotestosterone (DHT)-exposed animal model of PCOS as shown in a previous study [33]. Also, the present study reports an attenuating effect of AR blocker, flutamide on oxidative stress as well as cardiac and plasma endoglin. This outcome shows that AR activation is involved in the cardiac oxidative stress and increased cardiac and plasma endoglin in late gestational testosterone-exposed rats. However, there is no evidence herein to track the position of the two main outcomes (oxidative stress and Eng expression) relative to each other in connection to AR activation. Yet the findings in this study imply that endoglin, a co-receptor for TGF- β may interact with androgen to initiate intracellular signalling contributing to oxidative stress and that stimulation of AR itself might engender expression of Eng mRNA, suggesting that Eng expression is upstream of oxidative stress (Fig. 6).

A striking finding in this study is that acetate, a SCFA and also a known product of gut microbiota degradation of undigested fibers caused attenuation in cardiac oxidative stress and suppressed plasma and cardiac endoglin comparably with AR blockade in testosterone-exposed pregnant rats (Fig. 2). Evidence exists that SCFAs interact with G-protein coupled receptor (GPCR) family which is also believed to be activated by androgens [37–40]. This or the fact that acetate being a proven histone deacetylase (HDAC) inhibitor may double cross the transcriptional function of AR by promoting acetylation and inhibition of gene expression. Whatever be the case, this study gives evidence that although acetate might have its extra-AR mechanism(s) of resolving the testosterone-induced dysfunctions, it also possibly interacts with AR.

In conclusion, the study demonstrates that acetate, a SCFA could resolve the cardiac dysfunction along with oxidative stress and endoglin heightening caused by late gestational testosterone exposure with a

potency similar to flutamide, an AR blocker. This study indicates that gestational testosterone exposure could cause cardiac injury that is mediated by AR activation through an Eng-dependent defective antioxidant defense pathways. The findings in the present study also implies that acetate has therapeutic potential in conditions induced by testosterone exposure, particularly during pregnancy.

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Conflict of interest

The authors declare no conflict of interest.

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