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Sodium acetate and androgen receptor blockade improve gestational androgen excess-induced deteriorated glucose homeostasis and antioxidant defenses in rats: roles of adenosine deaminase and xanthine oxidase activities

Taofeek O. Usman[1,2,#], Emmanuel D. Areola[1,#], Olufunto O. Badmus[1,3,#], InKyeom Kim[4], Lawrence A. Olatunji[1]

[1]HOPE Cardiometabolic Research Team and Department of Physiology, College of Health Sciences, University of Ilorin, Nigeria

[2]Cardiovascular Unit, Department of Physiology, College of Health sciences, Osun State University, Osogbo, Nigeria

[3]Department of Public Health, Kwara State University, Malete, Nigeria

[4]Cardiovascular Research Institute and Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 700-842, Republic of Korea.

Running Title: Acetate and AR blockade improves glucoregulation and antioxidant barriers

[#]The authors contributed equally.

***Address correspondence to:**
Lawrence A. Olatunji, Ph.D.
Department of Physiology,
University of Ilorin,
P.M.B. 1515 Ilorin, 240003, Nigeria.
Tel: +2348035755360
E-mail: tunjilaw@unilorin.edu.ng

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ABSTRACT

Nutritional challenges and androgen excess have been implicated in the development of gestational diabetes and poor fetal outcome, but the mechanisms are not well delineated. The effects of short chain fatty acid (SCFA) on glucose dysmetabolism and poor fetal outcome induced by gestational androgen excess is also not known. We tested the hypothesis that blockade of androgen receptor (AR) and suppression of late gestational androgen excess prevents glucose dysmetabolism and poor fetal outcome through suppression of adenosine deaminase (ADA)/xanthine oxidase (XO) pathway. Twenty-four pregnant Wistar rats were treated (sc) with olive oil, testosterone propionate (0.5 mg/kg) singly or in combination with SCFA (sodium acetate; 200 mg/kg; *po*) or AR blocker (flutamide; 7.5 mg/kg; *po)* between gestational days 14 and 19. The results showed that late gestational androgen excess led to glucose deregulation, poor fetal outcome, increased plasma and hepatic free fatty acid and lactate dehydrogenase, liver function marker enzymes, malondialdehyde, uric acid, ADA and XO activities. Conversely, gestational androgen excess resulted in reduced body weight gain, visceral adiposity, plasma and hepatic anti-oxidant defenses (glutathione peroxidase, reduced glutathione/glutathione disulphide ratio, glucose-6-phosphate dehydrogenase, adenosine and nitric oxide). However, all these effects were ameliorated by either sodium acetate or flutamide treatment. The study demonstrates that suppression of testosterone by SCFA or AR blockade protects against glucose deregulation and poor fetal outcome by improvement of anti-oxidant defenses and replenishment of hepatic oxidative capacity through suppression of ADA/XO pathway. Hence, utility of SCFA should be encouraged for prevention of glucose dysmetabolism and poor fetal outcome.

Key words: Androgen receptor, gestational testosterone, gut microbiota metabolites, hepatic anti-oxidant, SCFA

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1. Introduction

Nutritional challenges and imbalance sex hormone exposure during pregnancy can contribute to disruption of glucose homeostasis and alter the postnatal phenotypic expression. Impaired insulin signaling results in insulin resistance (IR), a common metabolic phenotype, which has been associated with type II diabetes, impaired glucose tolerance, cardiovascular disease and liver disorders as well as polycystic ovary syndrome (PCOS) [1,2]. Sedentary life style together with western diet has been considered to contribute to the development of metabolic disorders. Considerable evidence also exists to implicate gestational exposure of animals to excess testosterone in metabolic defects and poor fetal outcome comparable to those with PCOS [3,4]. In experimental setting, sheep and rats exposed to excess gestational androgen developed IR that is associated with hyperinsulinaemia and liver disorder [5,6]. The underlying mechanisms resulting in the development of IR due to gestational exposure to excess androgens particularly in the late gestational period remains unclear, although activation of mineralocorticoid receptor has been previously implicated in our laboratory [7].

Adenosine, a regulatory nucleoside to which various defensive functions have been attributed, has anti-inflammatory, vasodilatory and antioxidant properties. Also, its ability to mitigate renin-angiotensin system together with afore stated roles confer a protective shield on cardiometabolic and renal functions[8-10].Adenosine deaminase (ADA) is a polymorphic purine enzyme, which catalyses conversion of adenosine to inosine. It is present in almost all mammalian cells and the activity has been reported to have prominence in lymphoid/immune cells and some metabolic tissues like liver and skeletal muscle. The activity of ADA has been correlated with inflammatory response through its ability to reduce adenosine [9,11]. Reduced serum activity of ADA has been observed in normal pregnancy compared to non-pregnant

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women, whereas, increased activity was observed in disorders of pregnancies such as preeclampsia [12]. Likewise, an increase in ADA activity was also noted in non-pregnant T2DM patients [13]. Xanthine oxidase (XO) catalyzes the last two steps of purine metabolism, modulating hypoxanthine to xanthine and then to uric acid [14]. Expression of XO is highest in the liver [15]. Enhanced XO activity might be responsible for the pro-oxidant property of uric acid and its negative impact on cardiometabolic disorders [16]. XO uses molecular oxygen as an electron acceptor, thus generating superoxide and other reactive oxygen species (ROS) as by-product and this is responsible for the pathological conditions implicated in hyperuricemia [16].

The liver plays pivotal regulatory function in glucose metabolism by uptake of glucose in the postprandial state and converting it to glycogen and triglyceride and production of glucose in the post-absorptive state by glycogenolysis and gluconeogenesis. Oxidative stress is a key player in the pathophysiology of non-alcoholic fatty liver disease (NAFLD), and in the development and progression of T2DM [17]. Contributory enzymes in oxidative stress are particularly abundant in the liver. ROS are products of cellular metabolism and are well recognized for their roles as both injurious and beneficial species. Although, ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and the hydroxyl radical, are generated as the byproducts of normal oxygen metabolism, they create oxidative damage by interacting with other biomolecules. When ROS generation is uncontrolled or impaired, it is termed oxidative stress, the excessive availability of oxidants, which activates signaling pathways and has been implicated in the development of organ damage in metabolic and cardiovascular diseases [18]. Antioxidants are molecules that can convert ROS into less reactive products, thus neutralizing them. Antioxidants can be enzymes such as glutathione peroxidase (GPx), glutathione reductase, and glutathione

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transferase, as well as non-enzymatic endogenous molecules, such as adenosine, glutathione (GSH), α-tocopherol, β-carotene, and ascorbic acid, [19]. Glucose-6-phosphate dehydrogenase (G6PD) is not just the cytosolic enzyme for the synthesis of pentose phosphate but also contributes considerably to the anti-oxidant defenses through glutathione system, by converting glutathione disulphide (GSSG) to its reduced form, GSH by GPx [20].

Short chain fatty acids (SCFAs) are produced from the fermentation of complex carbohydrates and/or digestible fibres largely by the colonic microbiota, especially anaerobic bacteria [21]. More than 90% of the SCFAs present in the colon are constituted by acetate, propionate, and butyrate [22]. Acetate constitutes approximately 65% of the SCFA composition produced in the colon and it partially eludes hepatic clearance to reach micromolar concentrations in the systemic circulation [23]. SCFA treatment has been reported to ameliorate airway disease, colitis, kidney disease and metabolic syndrome in diet-induced obesity in mice [24]. Reports suggest that elevated testosterone can affect SCFA negatively [25]. Reports also showed that acetate decreased the lipopolysaccharide-induced production of intracellular reactive oxygen species (ROS) concomitant with an increase in glutathione [26]. Elevated testosterone in women with PCOS has been associated with IR as well as altered gut microbiota. 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 metabolic phenotypes. We therefore tested the hypothesis that sodium acetate or blockade of androgen receptor and suppression of late gestational androgen excess would prevent glucose dysmetabolism and poor fetal outcome through suppression of ADA/XO/uric acid pathway.

2. Materials and Methods

2.1. Animals

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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 ethical review committee, University of Ilorin, Ilorin, Nigeria, and every effort was made to minimize both the number of animals used and their suffering. Pregnant female Wistar rats weighing 130–150 g, obtained from the Animal House, College of Health Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria, were used. The animals were housed in a well-ventilated room maintained under standard conditions of light, humidity, feeding and temperature. Rats had unrestricted access to standard rat chow and tap water. Twenty-four (24) pregnant rats were randomly assigned to vehicle (Ctr), testosterone-exposed (Tes), flutamide + testosterone (Tes+Flu) and acetate + testosterone (Tes+Ace) groups (n = 6/group).

2.2.Treatment

The animals were treated (sc) with olive oil, testosterone propionate (0.5 mg/kg) singly or in combination with SCFA (sodium acetate; 200 mg/kg; *po*) [27] or AR blocker (flutamide; 7.5 mg/kg; *po)* [28] between gestational days 14 and 19. The treatments were administered once daily. Oral glucose tolerance test (OGTT) was performed on gestational day 19, and the animals were euthanized on gestational day 20 after 12 hours overnight fast.

2.3. Glucose homeostasis

Oral glucose tolerance test (OGTT) was performed 24 hrs before the end of the experiment. Blood sample was obtained from the tail of each animal before glucose load (2 g/kg *bw*) (*po*) and then sequentially after 30, 60, 90 and 120 min. Terminal fasting blood glucose (FBG) was determined after the rats had 12 h overnight fast on the day of the sacrifice. Blood glucose levels were determined using a glucometer (ONETOUCH®-LifeScans, Inc., Milpitas,

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CA, USA). Glucose tolerance was expressed as a function of the area under the OGTT curve (AUC).

2.4. Sample preparation

At the end of the experimental period, animals were anesthetized with sodium pentobarbital (50 mg/kg, *ip*) after 12 hours overnight fast. Blood was collected by retro-orbital puncture into heparinzed and EDTA bottles. Blood samples were centrifuged at 3000 rpm for 5 min. Plasma was stored frozen until needed for biochemical assay.

2.5. Biochemical assay

Testosterone was determined using ELISA kit from Elabscience (Wuhan, China) while plasma insulin was determined using ELISA kit from Ray Biotechnology, Inc. (Georgia, USA). IR and pancreatic β-cell function were determined using the homeostasis model assessment for insulin resistance (HOMA-IR; (fasting insulin* fasting glucose)/22.5) and HOMA-β formula (20*fasting insulin/3.5-fasting glucose) respectively. Triglyceride/glucose index was determined using standard formula (Lin ((fasting insulin*fasting glucose)/2)).ADA, XO, UA, GPx, GSH, GSSG, G6PD, FFA, adenosine, lactate, lactate dehydrogenase (LDH), malondialdehyde (MDA), aspartate transaminase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were determined by standard colorimetric methods. Nitric oxide (NO) was measured by non-enzymatic colorimetric assay obtained from Oxford Biomedical Research Inc. (USA).

2.6. Statistical Analysis

All data were expressed as means ± SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) and Bonferroni's Post hoc analysis. Statistically significant differences were accepted at $p < 0.05$.

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3. Results

3.1. Effect of flutamide and acetate in excess late gestational testosterone treated rats on body weight change, regional body fat, liver weight and uterine weight

Testosterone exposure led to decrease in body weight (Table 1) and body weight change (figure 1a). Flutamide + testosterone exposure did not affect body weight change (Figure 1a) whereas acetate treatment with testosterone exposure led to increase in body weight change (Figure 1a). Late gestational testosterone exposure alone and flutamide + testosterone exposure led to decrease in regional body fat signifying a lipolytic response (Figure 1b).Whereas acetate + testosterone exposure did not affect the regional body fat, showing the anti-lipolytic action of acetate (Figure 1c). Also, testosterone exposure alone and flutamide+ testosterone exposure led to an increase in liver weight (Figure 1c) whereas, acetate treatment with testosterone exposure did not affect the liver weight (Figure 1c). The uterine weight was not affected by testosterone treatment while flutamide + testosterone and acetate + testosterone treatment led to a decrease in uterine weight when compared with the controls (Figure 1d). However, food intake was unchanged across all treatment groups.

3.2. Effect of flutamide and acetate in excess late gestational testosterone treated rats on pregnancy outcomes: fetal weight, fetal number, placental weight and fetal resorption

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Testosterone exposure only and flutamide + testosterone exposure led to a decrease in fetal weight (figure 2a). Acetate + testosterone exposure did not affect the fetal weight compared with control (Figure 2a). Testosterone exposure only and flutamide + testosterone exposure led to a decrease in placental weight (Figure 2b).Whereas acetate + testosterone exposure did not affect the placental weight compared with control (Figure 2b). Also, testosterone exposure led to decrease in fetal number (Figure 2c) while flutamide + testosterone exposure and acetate + testosterone exposure did not affect fetal number when compared with control (Figure 2c). Testosterone exposure also led to an increase in fetal resorption while flutamide + testosterone exposure and acetate +testosterone exposure did not present any resorption (Figure 2d).

3.3. Effect of flutamide and acetate in excess late gestational testosterone treated rats on terminal fasting glycaemia, postload glycaemia and lipid profile

Terminal fasting glycaemia was elevated in the testosterone only exposed group compared with control. It was however attenuated in flutamide + testosterone and acetate + testosterone exposed groups compared with testosterone only group (Table 2). 1-h postload glycaemia was increased in testosterone-treated group when compared with the control while flutamide + testosterone and acetate + testosterone treatments led to a decrease in 1-h postload glycaemia when compared with the testosterone-treated group (Figure 3a). Also, testosterone only exposed groups had an increased AUC of OGTT, which was attenuated by flutamide + testosterone and acetate + testosterone treatments (Figure 3b). Circulating triglyceride was not different between the control and testosterone groups. However, flutamide + testosterone and acetate + testosterone treatment led to decrease in circulating triglyceride when compared with

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both the control and testosterone-treated groups (Table 2). Hepatic triglyceride as well as circulating and hepatic HDL-cholesterol was unchanged across all treatment groups (Table 2).

3.4 Effect of flutamide and acetate in excess late gestational testosterone treated rats on IR, pancreatic β-cell function and fasting insulin level

There was increase in insulin resistance in testosterone only exposed group compared to control group. However, flutamide + testosterone exposure and acetate + testosterone exposure attenuated insulin resistance when compared with the testosterone exposed group (Figure 3d). β-cell dysfunction was increased in testosterone only exposed group compared with control group while it was reduced in both flutamide + testosterone and acetate + testosterone exposed groups when compared with testosterone only exposed group (Figure 3e). Fasting insulinemia was increased in testosterone only exposed group compared with control group. However, fasting insulinemia was reduced in flutamide + testosterone and acetate + testosterone exposed groups when compared with testosterone only exposed group (Figure 3c).TyG was not affected by all experimental groups when compared with control group (Figure 3f). Also, testosterone only exposure led to increase in plasma and hepatic FFA as a marker of lipolysis whereas, flutamide + testosterone and acetate + testosterone exposed groups had attenuated hepatic and plasma FFA (Figure 7a and d).

3.5. Effect of flutamide and acetate in excess late gestational testosterone treated rats on plasma and hepatic ADA and XO activity and UA

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Testosterone only exposure led to increase in plasma and hepatic ADA, XO and UA when compared with the control group. However, plasma and hepatic ADA, XO and UA were attenuated in flutamide + testosterone and acetate + testosterone exposed groups when compared with testosterone exposed group (Figure 4).

3.6. Effect of flutamide and acetate in excess late gestational testosterone treated rats on plasma and hepatic glutathione system

Plasma and hepatic GPx (Figures 5a and d), GSH (Table 3), GSH/GSSG ratio (Figures 5b and e) and G6PD (Figures 5c and f) were reduced in the testosterone-treated group when compared with the controls. However, flutamide + testosterone and acetate + testosterone exposure led to an increase in plasma and hepatic GPx, GSH, GSH/GSSG and plasma G6PD when compared with the testosterone-treated group (Figures 5a-5e, Table 3). Surprisingly, only flutamide + testosterone exposure led to increase in hepatic G6PD while acetate + testosterone treatment had no effect on hepatic G6PD when compared with testosterone-treated group (Figure 5f). On the other hand, testosterone treatment led to increase in plasma and hepatic GSSG level, which was reversed by flutamide or acetate treatment (Table 3).

3.7. Effect of flutamide and acetate in excess late gestational testosterone treated rats on plasma and hepatic adenosine, NO and MDA

Plasma adenosine was reduced in the testosterone-treated group when compared with the control group (Figure 6a) whereas there was no change in hepatic adenosine among the treatment

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groups (Figure 6d). However, there was an increase in adenosine in the flutamide + testosterone and acetate + testosterone groups when compared with the testosterone-treated group (Figure 6a).Both hepatic and plasma NO were reduced in testosterone only exposed group compared with the control group (Figures 6b and d). However, flutamide + testosterone and acetate + testosterone treatment led to increase in NO when compared with testosterone only group (Figures 6b and d). Plasma and hepatic MDA was increased in the testosterone-treated group when compared with the control while flutamide + testosterone and acetate + testosterone treatment led to decrease in MDA when compared with testosterone only group (Figures 6c and f).

3.8. Effect of flutamide and acetate in excess late gestational testosterone treated rats on plasma and hepatic oxidative capacity

Lactate being a marker of cell oxidative capacity did not change in the plasma in all experimental groups compared with control group (Figure 7b). However, hepatic lactate was depleted in testosterone-treated group compared with control group but was replenished in flutamide+ testosterone and acetate + testosterone exposed groups when compared with testosterone-treated group (Figure 7e). Both plasma and hepatic LDH activity increased in the testosterone only exposed group pointing to higher pyruvate production since lactate was depleted by same treatment. However, plasma and hepatic LDH activity was reduced in flutamide + testosterone and acetate + testosterone exposed groups when compared with testosterone group (Figures 7c and f).

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3.9. Effect of flutamide and acetate in excess late gestational testosterone treated rats on plasma and hepatic injury prognostic enzymes

In the testosterone only exposed group there was increase in all liver prognostic enzymes compared with the control. Flutamide + testosterone and acetate + testosterone exposure led to significant decrease in the prognostic scores of liver injury compared with testosterone only exposed groups (Figure 8).

3.10. Effect of flutamide and acetate in excess late gestational testosterone treated rats on plasma testosterone

Testosterone only exposure led to increase in plasma testosterone level when compared with control group. Flutamide + testosterone and strikingly acetate + testosterone exposure led to significant decrease in testosterone level compared with the testosterone only exposed group (Figure 9).

4.1. Discussion

The present study demonstrates that late gestational excess testosterone-induced glucose deregulation, IR and poor fetal outcome are accompanied by deteriorated circulating and hepatic antioxidant defenses and increased ADA, and XO activities. The study also shows that suppression of elevated testosterone by AR blockade or a SCFA, sodium acetate, improves or protects against deteriorated glucose homeostasis, IR and poor pregnancy outcome. The

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suppressive effects of AR blockade and SCFA on late gestational elevated testosterone are associated with improvement of circulating and hepatic antioxidant defenses and reduced circulating and hepatic activities of ADA and XO.

4.1. SCFA improves glucose and lipid homeostasis following late gestational testosterone exposure

A variety of studies point to the fact that metabolic perturbations especially glucose deregulation and adiposity are ubiquitous precursors of CVD, T2DM and hepatic diseases [1,2]. Also, studies reveal that altered metabolism during gestation may result in hyperglycemia and, potentially, gestational diabetes mellitus, an indication of future diabetes in the mother and exposure of offspring to risk of future diabetes [29]. The present study demonstrates that late gestational excess testosterone resulted in impaired maternal glucoregulation indicated by elevated AUC of OGTT, FFA, insulin, fasting glycaemia, pancreatic β-cell dysfunction and IR (Figure 3 and 7).In consonance, studies have shown that impaired glucose homeostasis and IR are attendant dysmetabolic effects of gestational excess androgen leading to poor fetal outcomes [3,4,30]. Despite the involvement of body weight gain and increased adiposity in the development of T2DM and other CMDs, our study attests to the weight gain and increased adiposity-independent metabolic derangement associated with gestational excess androgen (Figure 1a). Therefore, the finding in our study that flutamide (AR blocker) and acetate treatment corrected the metabolic derangements associated with gestational excess testosterone and replenished the body weight, agreeably shows that AR activation by testosterone is involved in the gestational excess testosterone-induced dysmetabolism and diminished body weight and acetate (SCFA) is able to prevent the AR-dependent effects. Besides, previous reports show that testosterone activates an alternative receptor, the orphaned G-protein coupled receptor

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(GPRC6A), to elicit its non-genomic actions leading especially to ROS generation [31] and SCFAs also interact with the GPCR family [32-35]. However, this study did not elucidate the probable contribution of this receptor to the effects realized. Likewise, the present study demonstrates that both AR blockade and SCFA treatment suppressed testosterone level in testosterone exposed pregnant rats. Based on previous reports by Hakim et al., of the deleterious effects of hyperandrogenism on fetal programming [4], the supression of gestational excess testosterone by SCFA as observed in this study has both maternal and fetal benefits especially the prevention of dysmetabolism.

SCFAs are products of bacterial degradation of undigested fibers and carbohydrates in the colon. They are known histone deacetylase (HDAC) inhibitors and therefore regulate various downstream metabolic processes including epigenetic modifications [36]. Acetate, a 2-Carbon fatty acid, is one out of the three main colonic SCFAs and it is the most abundant SCFA in the circulation[37]. Accumulating information from studies support the beneficial and protective role of SCFAs especially in models of metabolic derangement [38]. Aside restoration of glucose homeostasis in late gestational testosterone-treated rats, SCFA in this study elicited beneficial effects in lipid homeostasis as its treatment resulted in increased body weight despite regional fat, plasma FFA and hepatic FFA that is comparable with control group. This effect was not realized with AR blockade. Judging by this, SCFA (acetate) more effectively improves lipid dysmetabolism in adipocytes than AR blockade, but similarly improves glucose deregulation and FFA lipotoxicity in gestational testosterone exposure.

4.2. SCFA ameliorates lipotoxicity and improves anti-oxidant defense following late gestational testosterone exposure

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The present study revealed that late gestational excess testosterone caused elevation in both plasma and hepatic FFA (Figure 7a and d). Elevated circulating (spill over from adipocytes) and hepatic (deposition in non-adipocyte tissue) FFA indicates lipotoxicity, which is a known feature of obesity and precursor of T2DM [39-41]. FFA in the circulation can enter into hepatocytes and afterwards into the mitochondria through carnitine palmitoyltransferase 1a (CPT1a). In the mitochondria FFA is converted to Acetyl-CoA by β-oxidation producing NADH and $FADH_2$ which are oxidized in the electron transport chain (ETC). This process generates ROS and ATP [42]. Also, if FFA accumulates in the liver it can be converted to triglycerides and excess hepatic triglyceride causes liver steatosis. Lipotoxicity and ROS generation are intermediates that contribute to IR, liver steatosis and CMD. Hence, it is revealed in this study that gestational excess testosterone can induce a lipolytic IR since it reduced regional body fat and body weight change (Figure 1b) and generated FFA. Interestingly, both AR blockade and SCFA treatment in this study attenuated both plasma and hepatic FFA compared with testosterone only exposed group, showing that SCFA can effectively protect against late gestational excess androgen-mediated lipotoxicity and ROS as it has been shown in previous studies to inhibit lipolysis and lipogenesis in adipocytes [43].

Excess ROS or depleted anti-oxidant defenses has been implicated in the pathogenesis of various cardiometabolic diseases including T2DM[44]. There is evidence that maternal hyperglycemia from gestational diabetes mellitus (GDM) is associated with increased oxidative stress [45], which may also be involved in the pathophysiology of maternal and fetal complications of GDM [46].In the present study, late gestational excess testosterone enhanced hepatic MDA (Figure 6c), a product of lipid peroxidation and marker of oxidative stress, ROS generating enzymes and molecules while reducing the anti-oxidant defenses as observed in both

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the plasma and liver. The ADA-XO-UA pathway cleaves adenosine, an anti oxidant and anti inflammatory molecule, to uric acid a process that generates ROS and inhibits NO. In the present study, late gestational testosterone treatment led to elevated plasma and hepatic ADA, XO, UAand depleted adenosine and NO, a status that is known to promote ROS[47]and inflammation. Also, cellular anti-oxidant defenses is a complex interplay of cytosolic and mitochodrial reducing enzymes or factors. They scavenge ROS and superoxides in order to sustain cellular integrity.In the cytosol, G6PD is the rate limiting enzyme in the oxidative pentose phosphate pathway (PPP). It produces NADPH (being the major cyosolic source) [48]and resultantly GSH, which is utilized by GPx to reduce H_2O_2 to O_2 and H_2O producing GSSG.GSH/GSSG ratio has therefore been popularly used as an indicator of cellular redox status.Our results showed reduction in GSH/GSSG, GPx and G6PD with late gestational testosterone treatment. Again, AR blockade and SCFA treatment significantly attenuated the activities of ADA and XOand improved the glutathione antioxidant defense system. Evidence exist in previous studies of XO-induced ROS as the source of AR-mediated ROS generation [49]. Therefore, for the first time, this study implicated the ADA/XO pathway and the glutathione antioxidant system as the pivotal mechanisms through which late gestational excess testosterone elicits its deleterious effects via AR activation and that SCFA is able to ameliorate the AR-mediated dysfunction in these mechanisms.

Hepatic adenosine depletion and increased ADA and XO activity will activate hepatic macrophage [50,51] promoting inflammatory response and cell injury. The results of the present study revealed that gestational testosterone treatment induced liver injury associated with elevated plasma and hepatic liver disease prognostic enzymes (Figure 8). This together with

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increased liver weight in the same treatment supports inflammatory damage to hepatocytes. AR blockade and acetate treatment prevented the liver injury indicated by attenuation in liver prognostic enzymes (Figure 8). Although, only acetate normalized the liver weight. Therefore, AR hyperactivity in pregnancy might lead to liver damage through ROS generation and inflammatory response and for the first time, it is demonstrated herein that SCFAs possess therapeutic and protective potentials in AR-dependent hepatic injury.

4.3. SCFA attenuated the elevated oxidative process and improved the poor fetal outcome following late gestational testosterone exposure

The Liver is one of the most active organs and it largely determines the metabolic stand of the body. Hepatic injury via ROS-induced damage and inflammation will quickly compromise metabolic homeostasis. However, neglecting the redox system, diminished cellular oxidative capacity is sometimes a marker of metabolic diseases and elevated blood lactate is the culprit [52,53]. The present study revealed elevated hepatic and plasma LDH activity along with unaltered lactate in the plasma and reduced lactate in the liver with late gestational testosterone treatment (Figure 7). LDH catalyzes the reversible reaction that produces lactate from pyruvate in the cytosol. This reaction, when reversed, produces NADH and pyruvate which can be converted to Acetyl-CoA for oxidative processes in the mitochondria. The unaltered plasma lactate and reduced hepatic lactate and concomitant increase in LDH activity in this study shows that the reaction partially favours pyruvate production pointing to the possibility that mitochondrial function or oxidative capacity was not impaired. Moreso, studies have shown that elevated blood lactate is an indicator of oxidative insuffeciency which sometimes characterizes IR and T2DM[52,53] and this study revealed that gestational excess androgen did not result in this since blood lactate level was maintained by testosterone treatment compared with control.

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The fact that AR blockade restored hepatic lactate and reversed plasma and hepatic LDH activity from the testosterone-induced state shows that androgens and AR activation can increase LDH activity to produce pyruvate for the oxidative process in the mitochondria. This is in line with previous studies which showed that androgens increases mitochondrial ROS generation[54]. This study is however limited to arguing for the fact that excess androgen in pregnant rats results in metabolic derangements that is not related to cellular oxidative insuffeciency. Equally, acetate (SCFA) replenished hepatic lactate and reversed plasma and hepatic LDH activity from the testosterone-induced state. This shows that if SCFAs will not function through AR, they might be involved with a metabolic switch like AMP- activivated protein kinase.

As discussed above, the present study demonstrates that late gestational excess testosterone resulted in impaired maternal glucoregulation and hepatic injury, which are accompanied by increased ROS generating enzymes and depleted antioxidant defenses but without evidence of lipogenesis and oxidative incapacitation. Resultantly, and also in this study, late gestational excess testosterone induces poor pregnancy outcomes such as reduced fetal weight, placental weight, and fetal number and increased fetal resorption attesting to the deleterious effects of the metabolic derangements associated with gestational excess androgen. It is therefore plausible that gestational androgen-induced pregnancy outcomes are accompanied by metabolic perturbations and insights therein may open avenues for innovative preventive and therapeutic interventions. Strikingly, acetate treatment, like AR blockade, effectively averted the poor pregnancy outcomes that accompanied gestational excess testosterone. Although usage of acetate supplementation by pregnant women may be with caution, a previous study has explored the possibility of adjusting maternal diet to harness the ability of the intestinal microbiome to

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generate short chain fatty acids, including acetate, which could then exert protective effects on the offspring [55].

4.4. Summary

In summary, this study shows that the dysmetabolism associated with excess late gestational testosterone underlies a constellation of untoward pregnancy outcomes. This dysmetabolism is neither associated with increased body weight nor evidence of oxidative insuffeciency, but presents with activation of ADA/XO/UA pathway and depletion of G6PD-dependent anti oxidant defenses which are implicated in the effects of gestational androgen exposure, and can be plausible therapeutic targets. In this study, AR blockade and SCFA treatment resolves these pertubations revealing the wide involvement of AR in gestational excess testosterone metabolic effects and the remarkable potential of SCFA. However, since acetate, comparably with AR blockade, restored hepatic and plasma glucoregulation and redox status, alongside ameliorating the untoward pregnancy outcomes, SCFAs, being of greater dietary value than flutamide, might be a more tolerable non-toxic antidote to gestational excess androgen's deleterious effects.

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Conflict of interest

The authors have no conflict of interest to declare

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Figure Legends

Fig. 1. SCFA improves body weight gain (a), regional body fat (b), liver weight (c), and uterine weight (d) following late gestational testosterone exposure. Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed byone way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 2. SCFA improves fetal outcome following late gestational testosterone exposure. There was an improvement in fetal weight (a),placenta weight (b) and number of fetus (c) while resorption was not detected (d). Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 3. SCFA improves glucose homeostasis in pregnant rats following late gestational testosterone exposure. SCFA improved 1-hr postload glycemia (a), area under curve of oral glucose tolerance (b), fasting insulin level (c), HOMA-IR (d), HOMA-β (e) and the triglyceride/glucose index (f). Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 4. SCFA reverses the increase in plasma and hepatic levels of inflammatory markers observed in the Tes group. There was a reversal in plasma and hepatic levels of adenosine deaminase (ADA, a & d), xanthine oxidase (XO, b & e) and uric acid (UA, c & f).Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 5.SCFA improves both the plasma and hepatic glutathione systems when compared with the T group. There was an improvement in plasma and hepatic glutathione peroxidase (GPx, a & d), glutathione transferase/glutathione disulphide ratio (GSH/GSSG, b & e) and plasma glucose-6-phosphate dehydrogenase (G6PD, c). However, the hepatic G6PD was not affected by SCFA when compared with the Tes group (f).Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* T group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 6.SCFA improves plasma adenosine (a), plasma and hepatic nitric oxide (NO, b & e) and ameliorates plasma and hepatic malondialdehyde (MDA, c & f). Hepatic adenosine was however not affected by all treatments (d). Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

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Fig. 7.SCFA ameliorated plasma and hepatic free fatty acid (FFA, a&d) and lactate dehydrogenase (LDH, c & f) levels. There was an improvement in hepatic lactate levels (e) while the plasma level was unaffected by the treatments. Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 8.SCFA ameliorated both plasma and hepatic injury prognostic enzymes. There was a reversal in the plasma and hepatic levels of aspartate transaminase (AST, a & d), alkaline phosphatase (ALP, b & e) and gamma glutamyl transferase (GGT, c & f). Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, #p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 9.SCFA caused a reduction in circulating testosterone levels when compared with the Tes group. Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Fig. 10. Schematic diagram depicting the possible pathway through which SCFA or androgen receptor blocker interferes with the development of late gestational testosterone-induced cardiometabolic/oxidative disturbances. AR: Androgen receptor, XO: Xanthine oxidase, ADA: Adenosine deaminase, T2DM: Type II Diabetes Mellitus.

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Table 1: Body weight, food intake and fasting blood glucose

	Ctr	Tes	Tes+Flu	Tes+Ace
Body weight (g)	194.8 ± 13.6	172.2 ± 11.0*	178.8 ± 10.4	204.6 ± 2.9$^{\#}$
Food intake (g/kg/day)	99.5 ± 7.2	97.6 ± 6.9	94.5 ± 4.1	91.7 ± 1.2
Fasting blood glucose (mmol/l)	3.4 ± 0.1	4.2 ± 0.2*	3.2 ± 0.2$^{\#}$	3.3 ± 0.4$^{\#}$

Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

Table 2: Lipid parameters

	Ctr	Tes	Tes+Flu	Tes+Ace
Triglyceride				
Plasma (mg/dl)	84.0 ± 5.1	92.2 ± 5.0	35.5 ± 1.5*$^{\#}$	63.6 ± 3.2*$^{\#}$
Hepatic (mg/100g tissue)	120.1 ± 5.3	111.3 ± 10.1	93.8 ± 5.0	107.2 ± 5.9
HDL-cholesterol				
Plasma (mg/dl)	25.8 ± 0.5	26.0 ± 0.6	24.7 ± 0.4	26.7 ± 0.8
Hepatic (mg/100g tissue)	25.5 ± 0.7	26.0 ± 0.9	25.3 ± 0.8	24.3 ± 0.3

Values are expressed as mean ± SEM of 6 rats per group; *p<0.05 *vs* control, $^{\#}$p<0.05 *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

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Table 3: Plasma and hepatic reduced glutathione and oxidized glutathione disulphide

	Ctr	Tes	Tes+Flu	Tes+Ace
GSH				
Plasma (U/l)	6.4 ± 0.8	2.5 ± 0.2*	5.3 ± 0.6$^{\#}$	5.6 ± 0.5$^{\#}$
Hepatic (U/mg protein)	5.6 ± 0.5	1.6 ± 0.6*	5.6 ± 0.5$^{\#}$	5.3 ± 0.9$^{\#}$
GSSG				
Plasma (U/l)	0.14 ± 0.05	1.47 ± 0.44*	0.12 ± 0.05$^{\#}$	0.17 ± 0.03$^{\#}$
Hepatic (U/mg protein)	0.27 ± 0.14	6.70 ± 1.27*	1.21 ± 0.69$^{\#}$	3.05 ± 1.42$^{\#}$

Values are expressed as mean ± SEM of 6 rats per group; *$p<0.05$ *vs* control, $^{\#}p<0.05$ *vs* Tes group. Data were analyzed by one way ANOVA and Bonferroni's *Post hoc* analysis; GSH; reduced glutathione, GSSG; glutathione disulphide, Ctr; control, Tes; testosterone, Flu; flutamide, Ace; acetate.

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Sodium acetate and androgen receptor blockade improve gestational androgen excess-induced deteriorated glucose homeostasis and antioxidant defenses in rats: roles of adenosine deaminase and xanthine oxidase activities

Highlights

The findings of this study are:

- Suppression of testosterone by short chain fatty acid (SCFA) or androgen receptor (AR) blockade protects against glucose deregulation and poor fetal outcome in pregnant rats.
- The effects of SCFA and AR blockade observed in this study are due to improvement of anti-oxidant defenses through suppression of adenosine deaminase/xanthine oxidase pathway.
- Therefore, utility of SCFA should be encouraged for prevention of glucose dysmetabolism and poor fetal outcome.

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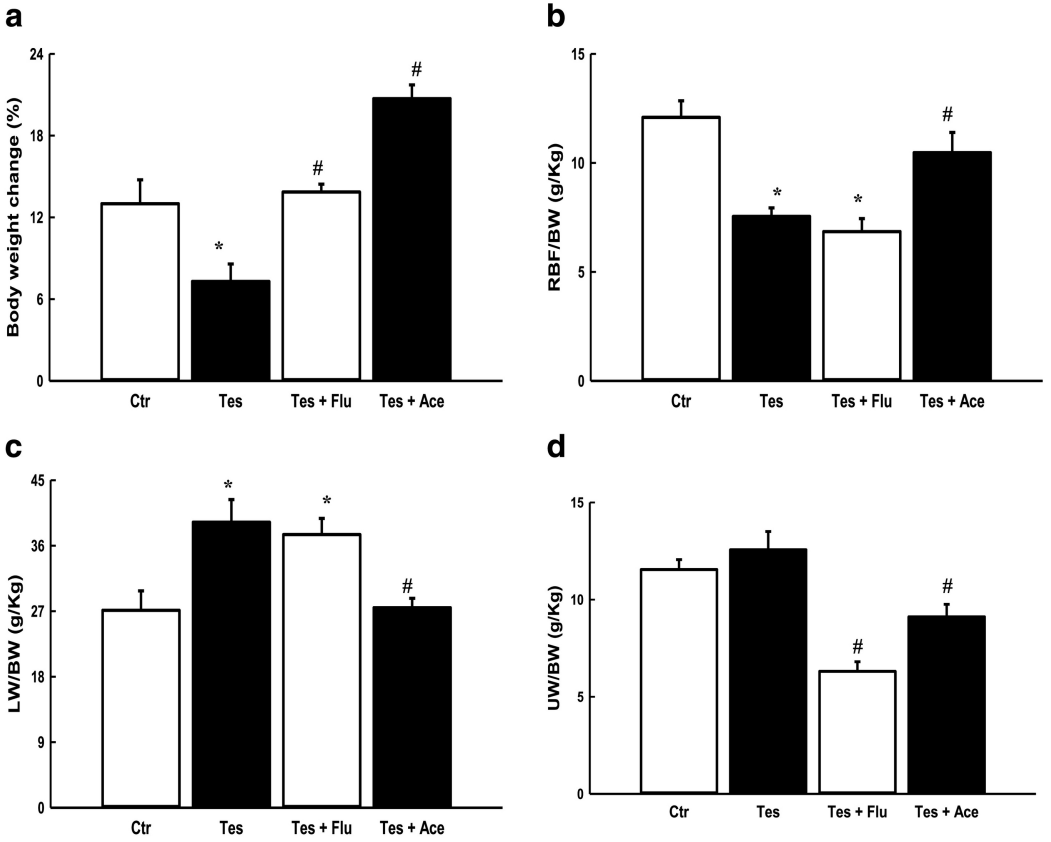

a
Body weight change (%)
24
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
RBF/BW (g/Kg)
15
10
5
0
*
*
#
Ctr
Tes
Tes + Flu
Tes + Ace
c
LW/BW (g/Kg)
45
36
27
18
9
0
*
*
#
Ctr
Tes
Tes + Flu
Tes + Ace
d
UW/BW (g/Kg)
15
10
5
0
#
#
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 1

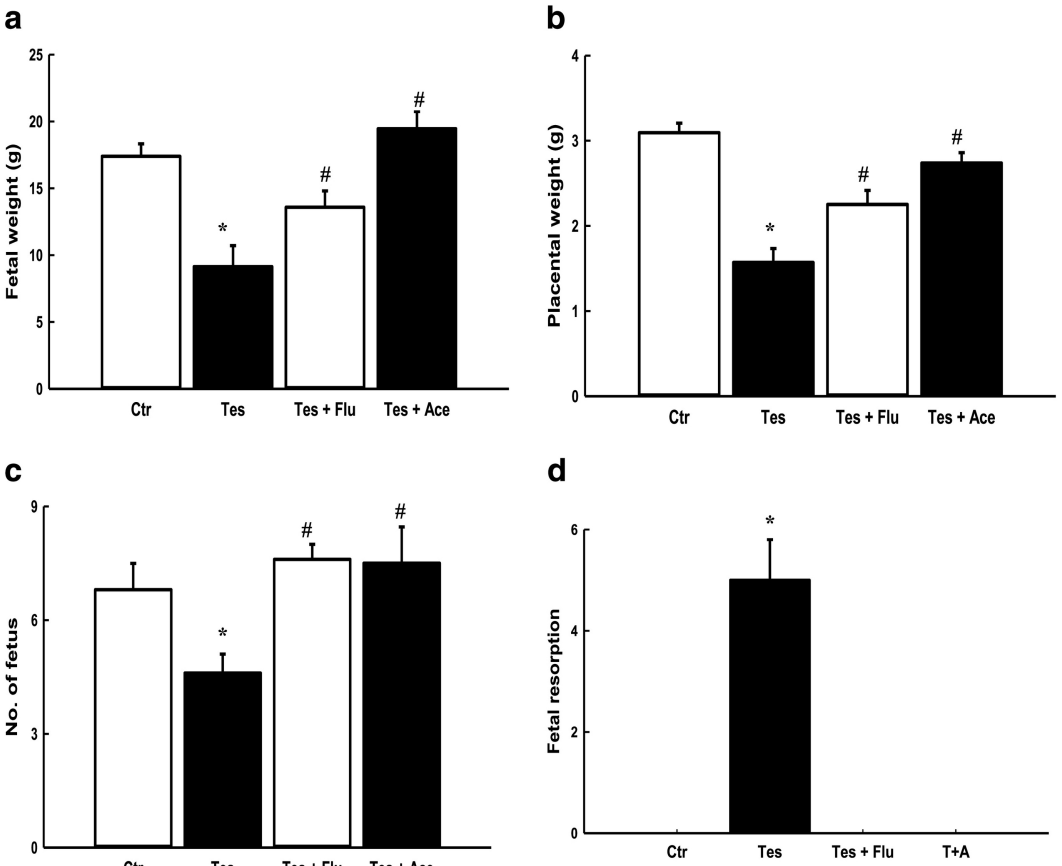

a
Fetal weight (g)
25
20
15
10
5
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
Placental weight (g)
4
3
2
1
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
c
No. of fetus
9
6
3
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
d
Fetal resorption
6
4
2
0
*
Ctr
Tes
Tes + Flu
T+A

Figure 2

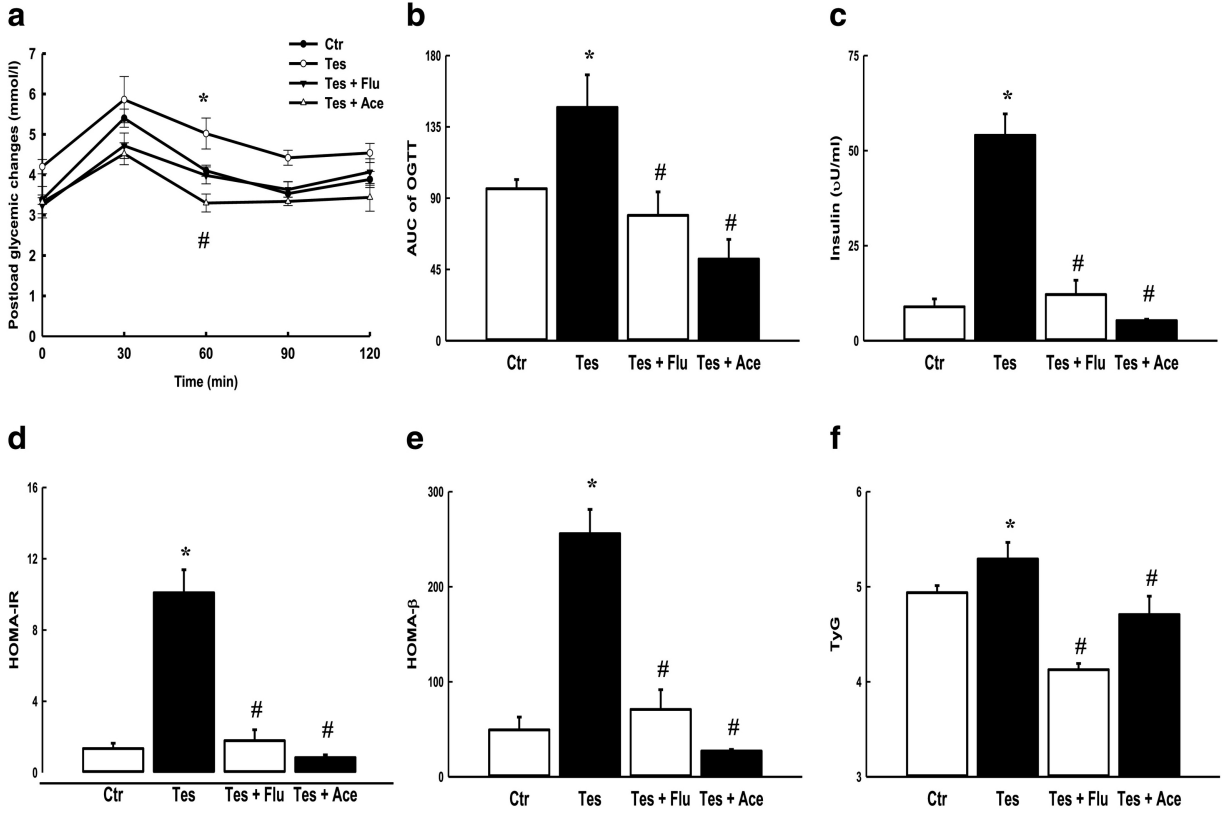

a
Ctr
Tes
Tes + Flu
Tes + Ace
Postload glycemic changes (mmol/l)
Time (min)
b
AUC of OGTT
c
Insulin (ωU/ml)
d
HOMA-IR
e
HOMA-β
f
TyG
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 3

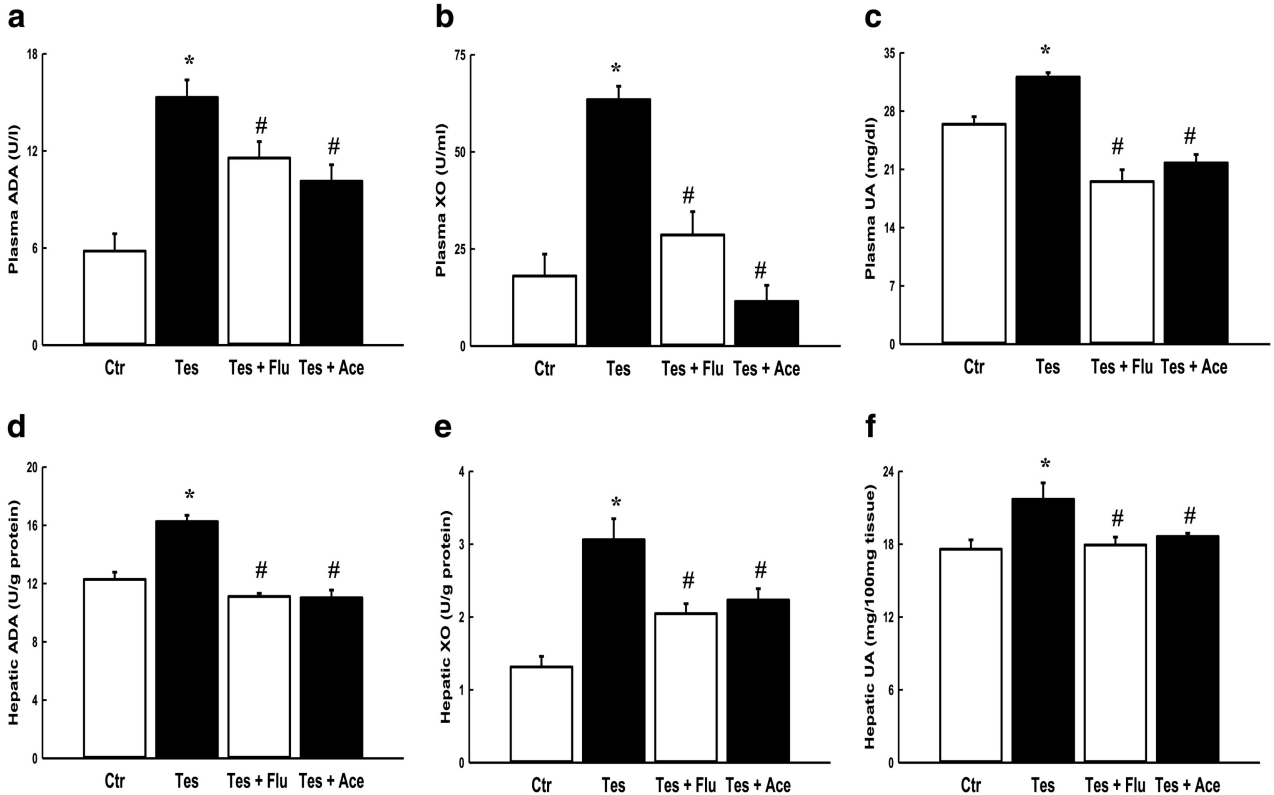

a
Plasma ADA (U/l)
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
Plasma XO (U/ml)
75
50
25
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
c
Plasma UA (mg/dl)
35
28
21
14
7
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
d
Hepatic ADA (U/g protein)
20
16
12
8
4
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
e
Hepatic XO (U/g protein)
4
3
2
1
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
f
Hepatic UA (mg/100mg tissue)
24
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 4

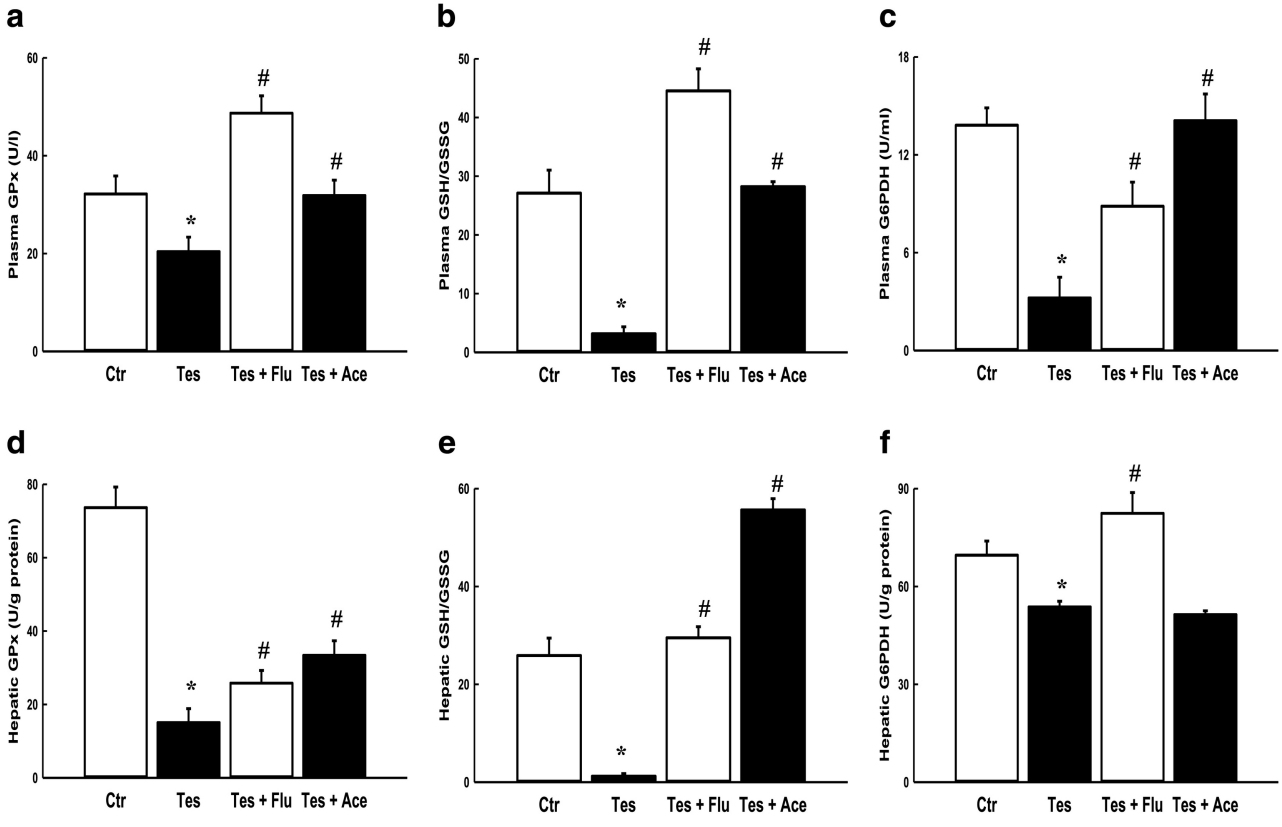

a
Plasma GPx (U/l)
60
40
20
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
Plasma GSH/GSSG
50
40
30
20
10
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
c
Plasma G6PDH (U/ml)
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
d
Hepatic GPx (U/g protein)
80
60
40
20
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
e
Hepatic GSH/GSSG
60
40
20
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
f
Hepatic G6PDH (U/g protein)
90
60
30
0
*
#
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 5

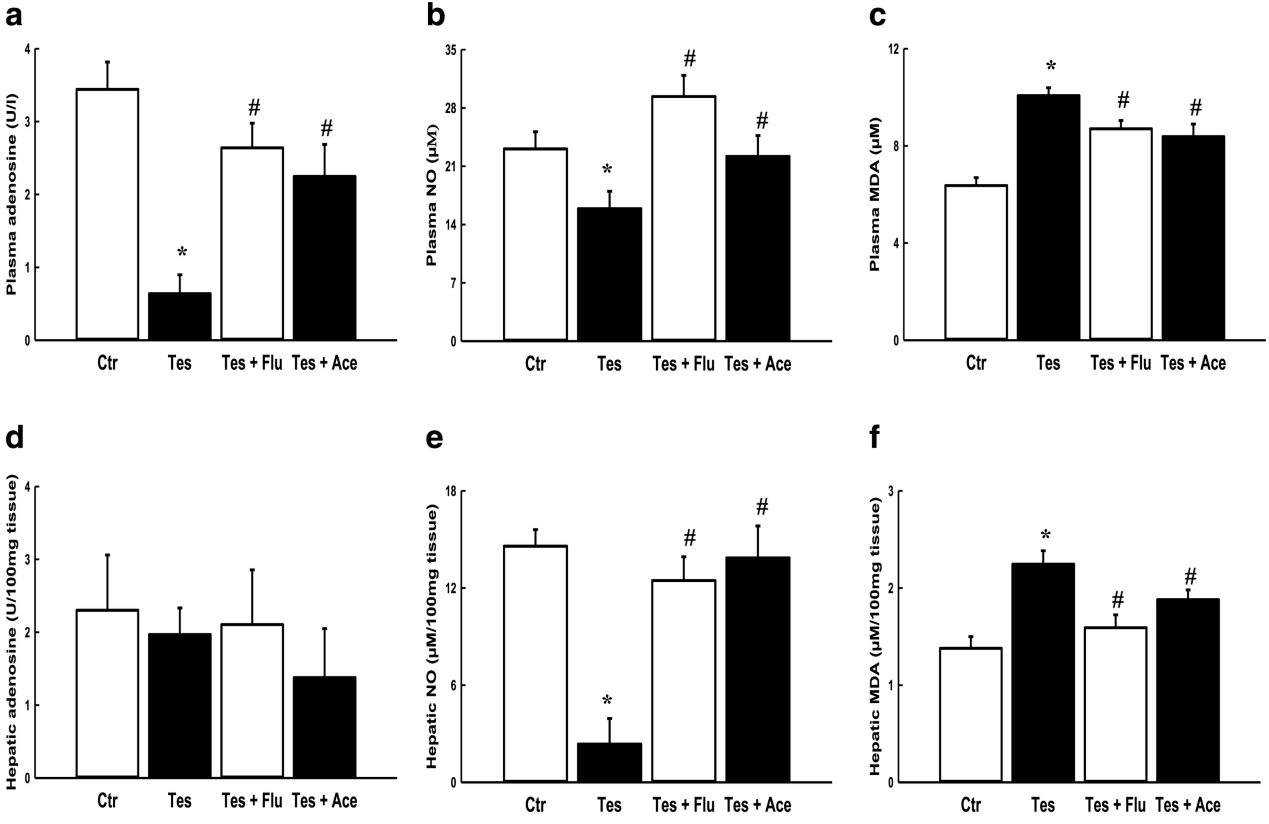

a
Plasma adenosine (U/l)
4
3
2
1
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
Plasma NO (μM)
35
28
21
14
7
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
c
Plasma MDA (μM)
12
8
4
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
d
Hepatic adenosine (U/100mg tissue)
4
3
2
1
0
Ctr
Tes
Tes + Flu
Tes + Ace
e
Hepatic NO (μM/100mg tissue)
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
f
Hepatic MDA (μM/100mg tissue)
3
2
1
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 6

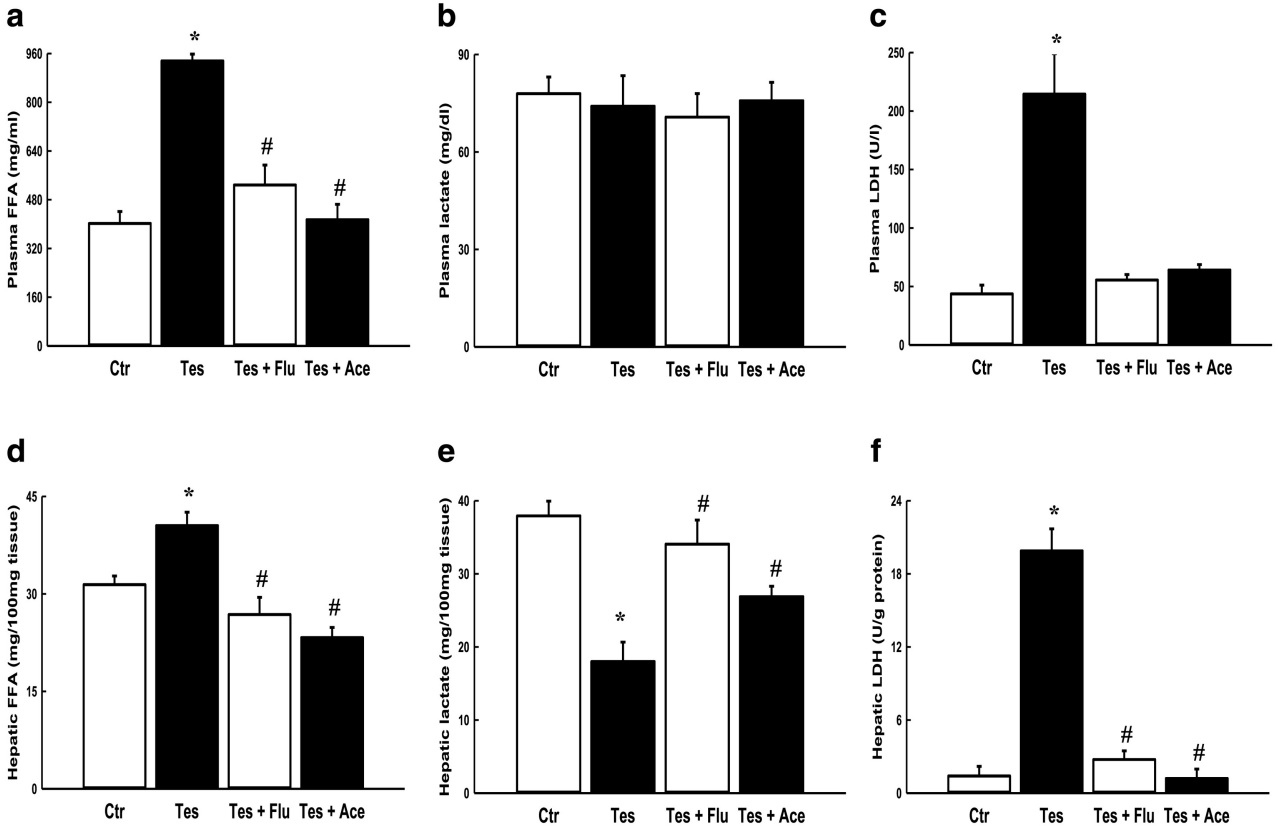

a
Plasma FFA (mg/ml)
960
800
640
480
320
160
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
Plasma lactate (mg/dl)
90
60
30
0
Ctr
Tes
Tes + Flu
Tes + Ace
c
Plasma LDH (U/l)
250
200
150
100
50
0
*
Ctr
Tes
Tes + Flu
Tes + Ace
d
Hepatic FFA (mg/100mg tissue)
45
30
15
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
e
Hepatic lactate (mg/100mg tissue)
40
30
20
10
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
f
Hepatic LDH (U/g protein)
24
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 7

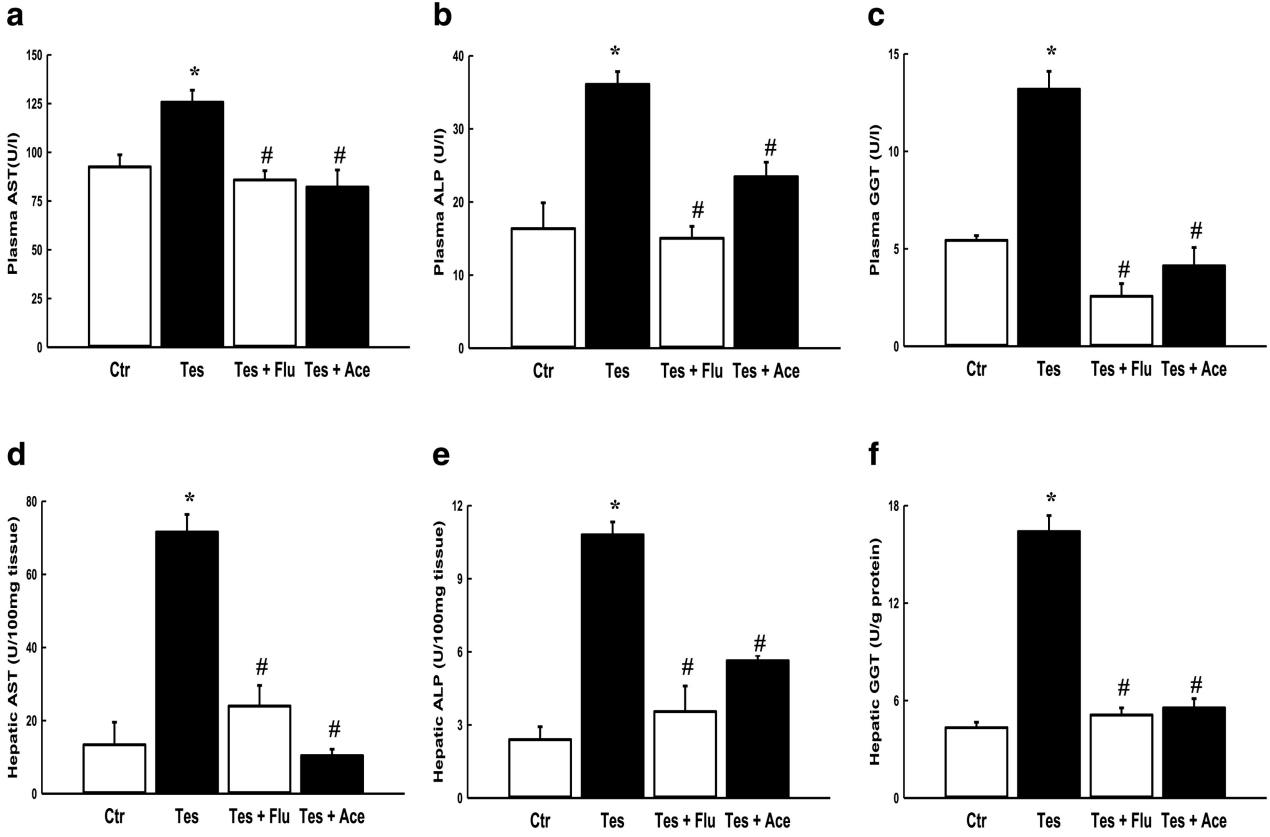

a
Plasma AST(U/l)
150
125
100
75
50
25
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
b
Plasma ALP (U/l)
40
30
20
10
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
c
Plasma GGT (U/l)
15
10
5
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
d
Hepatic AST (U/100mg tissue)
80
60
40
20
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
e
Hepatic ALP (U/100mg tissue)
12
9
6
3
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace
f
Hepatic GGT (U/g protein)
18
12
6
0
*
#
#
Ctr
Tes
Tes + Flu
Tes + Ace

Figure 8

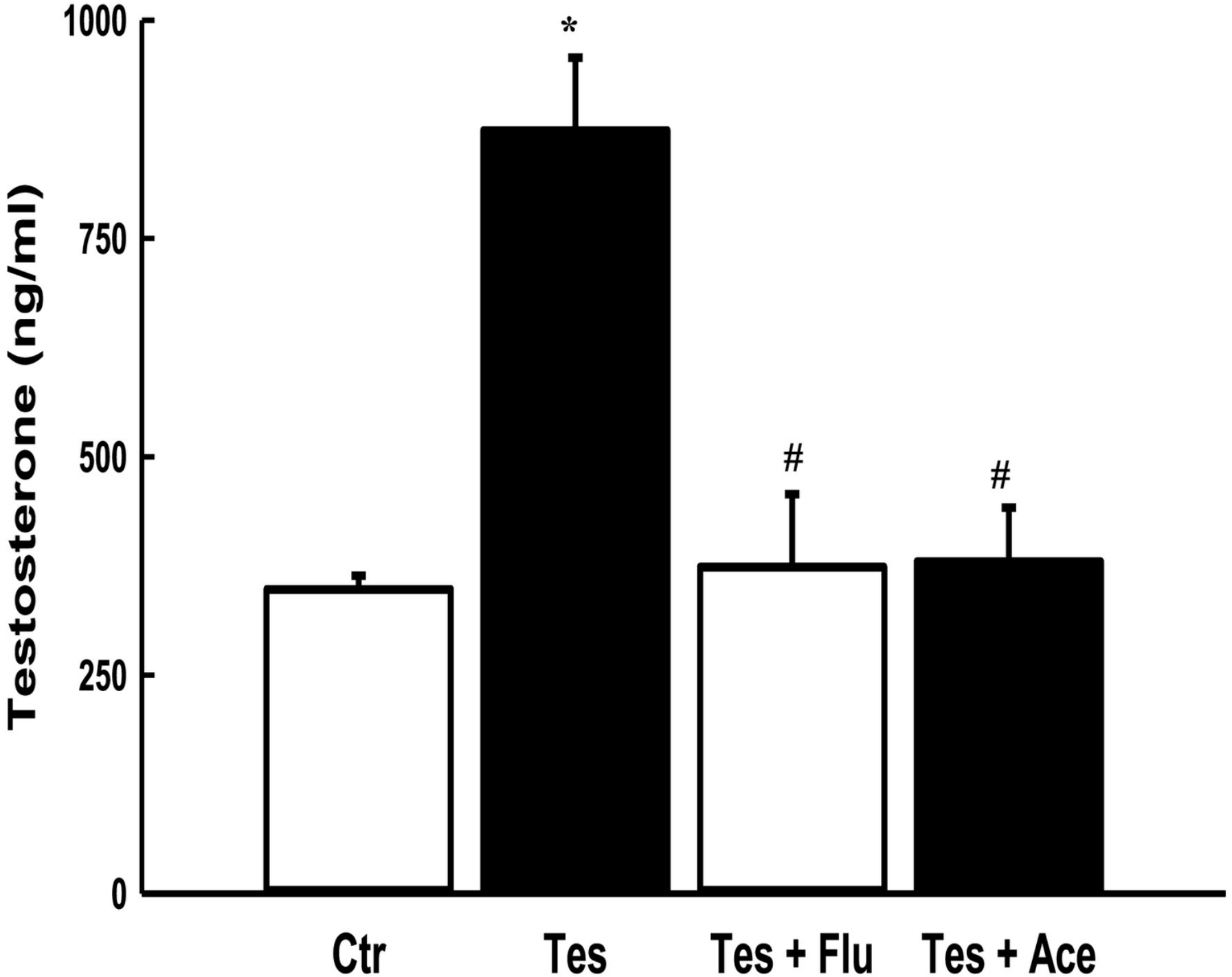


Figure 9

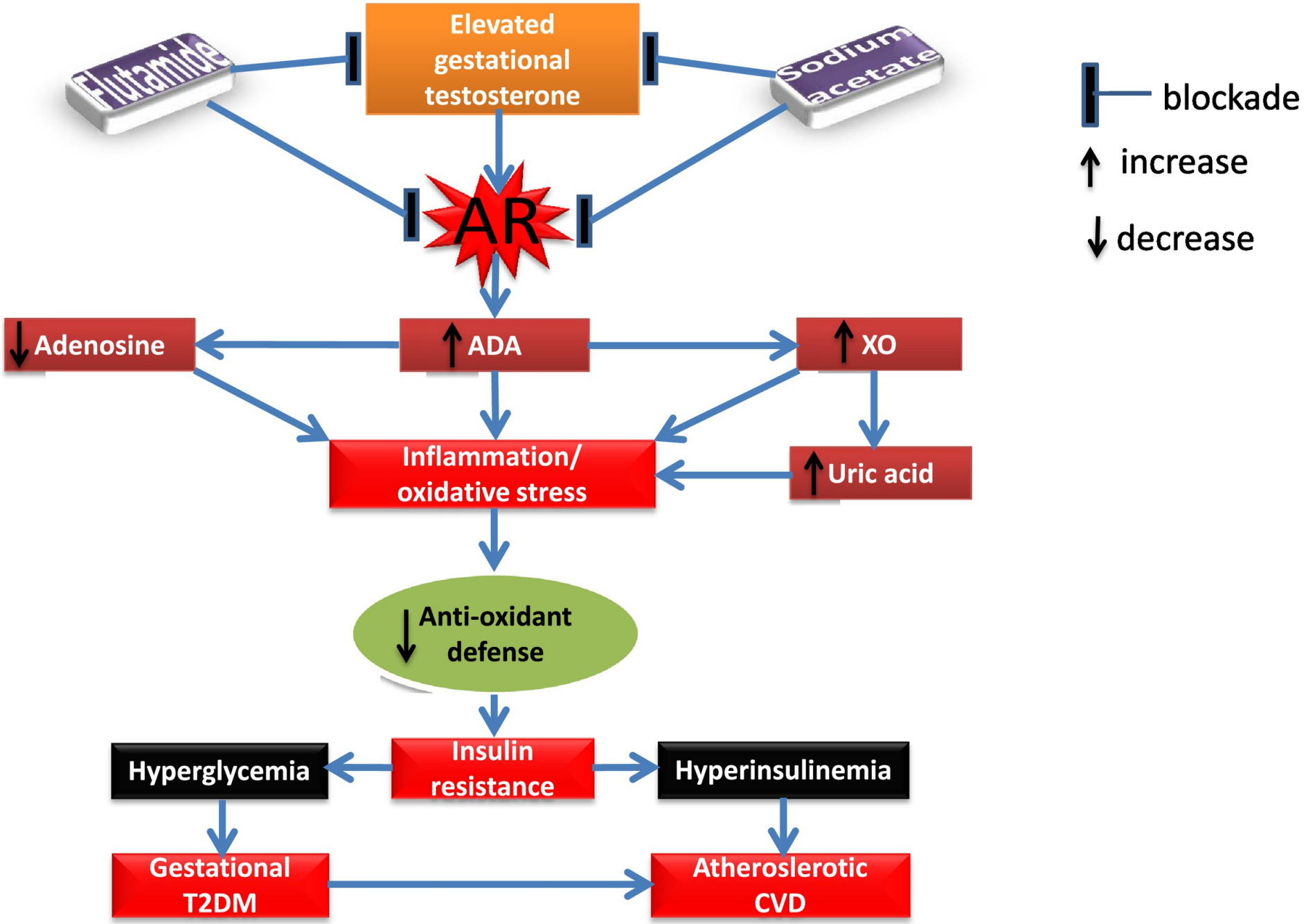

Flutamide
Elevated gestational testosterone
Sodium acetate
AR
blockade
increase
decrease
Adenosine
ADA
XO
Inflammation/ oxidative stress
Uric acid
Anti-oxidant defense
Hyperglycemia
Insulin resistance
Hyperinsulinemia
Gestational T2DM
Atherosclerotic CVD

Figure 10