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Sildenafil augments fetal weight and placental adiponectin in gestational testosterone-induced glucose intolerant rats

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ABSTRACT

Testosterone induces intra-uterine growth restriction (IUGR) with maternal glucose dysregulation and oxidant release in various tissues. Adiponectin, which modulates the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) signaling is expressed in the placenta and affects fetal growth. Sildenafil, a phosphodiesterase type 5 inhibitor (PDE5i), used mainly in erectile dysfunction has been widely studied as a plausible pharmacologic candidate in IUGR. Therefore, the present study sought to determine the effect of PDE5i on placental adiponectin/Nrf2 pathway in gestational testosterone-induced impaired glucose tolerance and fetal growth. Fifteen pregnant Wistar rats were allotted into three groups (n = 5/group) receiving vehicles (Ctr; distilled water and olive oil), testosterone propionate (Tes; 3.0 mg/kg; sc) or combination of testosterone propionate (3.0 mg/kg; sc) and sildenafil (50.0 mg/kg; *po*) from gestational day 14–19. On gestational day 20, plasma and placenta homogenates were obtained for biochemical analysis as well as fetal biometry. Pregnant rats exposed to testosterone had 4-fold increase in circulating testosterone compared with control (20.9 ± 2.8 vs 5.1 ± 1.7 ng/mL; $p < 0.05$) whereas placenta testosterone levels were similar in testosterone- and vehicle-treated rats. Exposure to gestational testosterone caused reduction in fetal and placental weights, placental Nrf2 and adiponectin. Moreover, impaired glucose tolerance, elevated plasma triglyceride-glucose (TyG) index, placental triglyceride, total cholesterol, lactate, malondialdehyde and alanine aminotransferase were observed in testosterone-exposed rats. Treatment with sildenafil improved glucose tolerance, plasma TyG index, fetal and placental weights and reversed placental adiponectin in testosterone-exposed pregnant rats without any effect on placental Nrf2. Therefore, in testosterone-exposed rats, sildenafil improves impaired glucose tolerance, poor fetal outcome which is accompanied by augmented placental adiponectin regardless of depressed Nrf2.

1. Introduction

Intra-uterine growth restriction (IUGR) and low birth weight are caused by intra-uterine environmental perturbations. The prevalence of IUGR ranges in different parts of the world with the lowest lower limit of 5% in the United States and the highest upper limit of 55 % in South Central Asia [1,2]. Recently, the development of cardiovascular and cardiometabolic diseases in later life has been linked to occurrence of IUGR [3]. This therefore necessitated studying its etiology and endeavouring to identify suitable pharmacologic candidate for treatment. Human studies have shown that hypoxia, obesity, nicotine and nutritional restrictions are among the identified common causes of IUGR [4–7]. Nevertheless and relevant to the aim of the present study, discoveries have shown that high circulating maternal testosterone especially at late pregnancy is negatively associated with birth size outcome [8]. A decrease of 160 g was realized when circulating testosterone increased at 17 weeks gestation [8]. It is fascinating that studies have revealed elevated fetal nutritional deprivation in conditions with androgen excess [9] which shows that there is possibility of sub-optimal feto-placental vascularization or exchange during

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gestational hyperandrogenism. Also, reports of human studies show that elevated androgen concentrations and the increased proportion of small for gestational age newborns and IUGR have been linked to PCOS pregnancy and preeclampsia respectively [10,11]. Evidences from animal studies have also contributed to the knowledge available as evidence for the impact of excess gestational testosterone on IUGR. Testosterone exposure in mother sheep reduced weight of both male and female newborns [12].

Late pregnancy cardiometabolic syndrome and preeclampsia are lethal and increasingly studied. The occurrence of preeclamptic symptoms is linked to gestational hyperandrogenamia which is associated with maternal tissue derangement and poor pregnancy outcome [13,14]. However, the mechanism (s) involved in androgen-induced gestational maternal and fetal disorders is still unclear. Studies from our laboratory that focused on maternal effects of gestational excess androgen have shown various tissue metabolic derangements. Usman et al. showed that testosterone exposure in late pregnancy caused glucose dysregulation and hepatic lipid disturbances in rats [15]. Also, Olatunji et al. showed that testosterone in late pregnancy is linked to elevated cardiac endoglin and depressed glucose-6-phosphate dehydrogenase-dependent antioxidant defense in the heart. This cardiac effect of gestational excess androgen was alleviated by flutamide, an androgen receptor (AR) blocker [16] showing that AR signaling (genomic or non-genomic) is involved in gestational testosterone-induced cardiac metabolic disturbance and endoglin-dependent fibrotic response. Usman et al. [15] and Olatunji et al. [16] verify that maternal tissues are unsafe in gestational hyperandrogenemic milieu and that the maternal tissue derangement caused by gestational excess androgen is associated with poor fetal outcome [15]. This puts placenta, the feto-maternal tissue interface in perspective. Therefore, studying placental metabolic alterations in hyperandrogenemic milieu might answer some of the questions on how excess androgens during pregnancy can lead to deficient fetal growth.

Findings have shown that among the ways by which androgens impact tissues, the release of oxidants is remarkable. It is agreeable that excess androgens in females can result in tissue oxidative stress either by enhancing oxidant release or depressing cellular antioxidant defense. Redox imbalance usually causes protein malfunction which can impair protein-fascilitated transport of essential materials across membranes. Therefore, studying redox responses in the placenta, an organ that provides transport interface for fetal nourishment, during gestational exposure to excess testosterone is reasonable for the verification of the mechanism involved in androgen-induced intrauterine growth restriction. Redox imbalance is evidently sufficient to result in poor placental development and function via inflammation, mitochondrial dysfunction, and lipid dysmetabolism. A major intracellular mechanism of maintaining redox balance that is stimulated by reactive oxygen species (ROS) is the Nrf2 signaling which leads to the production of intracellular antioxidants like superoxide dismutase, catalase, glutathione among others [17]. In metabolic syndrome, it is believed that depressed Nrf2 translocation is causative in the associated oxidative stress and mitochondrial dysfunction [18]. A study revealed that during angiotensin receptor blockade, improved Nrf2 signaling was associated with enhanced aconitase activity. Aconitase, a mitochondrial enzyme sensitive to cellular oxidants is growingly studied as a marker of mitochondrial dysfunction and has been negatively correlated with placental lipid accumulation. In addition, expression of adiponectin, a key anti-inflammatory adipokine and its receptors have also been characterized in the placenta and recent novel findings show that adiponectin impacts fetal growth but the mechanism is yet to be elucidated [19]. Some studies showed that adiponectin inhibits placental proliferation and nutrient transport whereas other studies showed that adiponectin levels are positively correlated with fetal weight [20]. However, adiponectin modulates Nrf2 signaling [21], positively correlates with aconitase activity and has anti-inflammatory and antioxidant effects in tissues of the body.

Sildenafil is a phosphodiesterase type 5 inhibitor (PDE5i) used mainly for male erectile dysfunction. By potentiating nitric oxide action through sparing of cyclic guanine monophosphate (cGMP), sildenafil administration leads to a strong vasodilation [22]. This drug is therefore used in other conditions like pulmonary hypertension and in athletes at high altitude [23,24]. Also, it was found to protect the mitochondrial function of hypoxic myocardial cells [25] and improve maternal and fetal health [26]. How PDE5 inhibitors may combat oxidative stress has been extensively studied in the kidney. A review showed that findings from several studies indicate that PDE5 inhibitors have antioxidative action and averts renal injury via various mechanisms such as enhancing mitochondria biogenesis, stimulation of eNOS improving medullary blood flow reversing Bcl2/Bax ratio, ERK phosphorylation, regulation of PI3K/Akt and NF-KB pathway, preventing endothelial damage and increasing podocyte progenitor cells [27]. Also, a study shows that PDE5 inhibitors improve renal function by increasing antioxidant expression in the kidney [28].

In the field of Obstetrics, many disease of the fetus are linked to poor placental vascularization and it was hypothesized that sildenafil might improve placental blood flow and metabolism [29]. However, several scientists have dared to experiment the potency of sildenafil relating to utero-placental circulation in both animal and human studies. Some studies show that sildenafil can improve maternal-fetal exchanges [30,31], whereas other studies reported insignificant effect on fetal growth [32] and adverse offspring cardiometabolic effects [33]. Nevertheless, reports also show that women that take sildenafil to resolve cardiovascular indications during pregnancy lacked adverse fetal teratogenic effect that can be linked to the drug [34,35]. Therefore, the present study sought to investigate the effect of sildenafil on placental adiponectin/Nrf2 pathway in impaired glucose tolerance and fetal growth caused by gestational testosterone exposure.

2. Materials and methods

2.1. Animals

The study was carried out in agreement with the guidelines of the National Institutes of Health Guide for the Care of Laboratory Animals in submission to approval by the Ethical Review Committee, University of Ilorin, Ilorin, Nigeria. Minimal number of animals and suffering of the animals obtained for experiment were achieved. Healthy female Wistar rats of ten (10) weeks old were obtained from College of Health Sciences Animal House, University of Ilorin, Ilorin, Nigeria. The animals were housed with good ventilation in a room kept under standard conditions of temperature (20–25 °C), humidity (30–50 %) and light (normal 24 h light-dark cycle). Animals weighing between 130 g–150 g were made pregnant and given access to standard rat chow and tap water without restriction. Fifteen (15) pregnant rats were assigned randomly to different groups of five (5) animals per group which received olive oil (control), testosterone propionate (Chemical name: [(8*R*,9*S*,10*R*,13*S*,14*S*,17*S*)-10,13-dimethyl-3-oxo-1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-17-yl] propanoate; chemical formula: $C_{22}H_{32}O_3$; CAS number: 57-85-2) and testosterone propionate with sildenafil (Source: Viagra®, Pfizer; purity: 99.7%; chemical name: 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulphonyl]-4methypiperazine citrate; Chemical formula: $C_{22}H_{30}N_6O_4S\bullet C_6H_8O_7$; CAS number: 139755-83-2, citrate: 171599-83-0).

2.2. Treatment dose and route of administration

Pregnant animals were treated with olive oil (sc) and distilled water (0.3 ml/day orally) in control group (Ctr), testosterone propionate diluted in olive oil (3.0 mg/kg; sc) singly in testosterone-exposed group (Tes) or in combination with sildenafil (50.0 mg/kg; *po*) in testosterone with sildenafil group (Tes + PDE5) from gestational days 14–19. Sildenafil was diluted in distilled water and testosterone in olive oil.

2.3. Glucose tolerance and metabolic disturbance assessment

Twenty-four hours (24) hours before the end of the experiment, oral glucose tolerance test (OGTT) was carried out with blood samples of approximately 20 μL obtained from the tail of each animal. Before glucose load (2 g/kg *bw*) (*po*) and then in succession after 30, 60, 90 and 120 min. of post-load period, blood glucose levels were determined using a glucometer (ONETOUCH®-LifeScans, Inc., Milpitas, CA, USA). Glucose tolerance was taken as a function of the area under the curve of OGTT (AUC). Metabolic disturbance was assessed by two prognostic scores, the triglyceride-glucose index (TyG) which was determined using standard formula (Lin ((Triglyceride (mg/dL)*fasting glucose (mg/dL)/2)) and the triglyceride-high density lipoprotein ratio (TG/HDL). The fasting insulin, TG and HDL levels were obtained by methods described in the biochemical assay section, whereas, glucose level was obtained with glucometer (ONETOUCH®-LifeScans, Inc., Milpitas, CA, USA) as described above.

2.4. Tissue and plasma sample preparation

On gestational day 20, after 12 h over night fast, pregnant animals were anesthetized with sodium pentobarbital (50 mg/kg, *ip*). Blood was collected by cardiac puncture into heparinzed sample bottles. Blood samples were centrifuged at 3000 rpm for 5 min. Plasma was stored frozen until needed for biochemical assay. Placenta from each pregnant animal were homogenized and centrifuged at 3000 rpm for 15 min. The tissue homogenates were obtained and kept for biochemical analysis.

2.5. Fetal and placental outcome

Fetuses were separated from placenta and both were weighed with digital weighing scale to obtain fetal weight and placental weight per group and fetal and placental weight per fetus.

2.6. Placental efficiency

Placental efficiency were determined by ratio of fetal weight to placental weight

2.7. Biochemical assay

2.7.1. Circulating and placental testosterone

Testosterone level was determined in plasma samples and placental homogenates using ELISA kit from Elabscience (Wuhan, China) with a sensitivity of 0.01 nmol/L, detection range of 0.01–0.70 nmol/L and the specificity is recognition of testosterone in sample. No significant cross-reactivity or interference between testosterone and analogues was observed and coefficient of variation is <10 %.

2.7.2. Placenta Nrf2

Placenta Nrf2 expression was determined in placental homogenates using ELISA kit (Elabscience, Wuhan, China; sensitivity: 9.38 pg/mL; detection range: 15.63–1000 pg/mL; specificity: rat Nfr2 recognition in samples. No significant cross-reactivity or interference between rat Nrf2 and analogues and coefficient of variation is <10 %). 100 μL of sample solution (placental homogenates from control, Tes and Tes + PDE5i groups) was added to well and incubated for 90 min at 37 °C. The liquid was removed. Biotinylated detection Ab was added and incubated for 1 h at 37 °C. After aspirating and washing 3 times, HRP conjugate (100 μL) was added and incubated for 30 min at 37 °C. Then 90 μL of substrate reagent was added and incubated for 15 min at 37 °C. Stop solution (50 μL) was added and optical density was read at 450 nm immediately.

2.7.3. Placenta cGMP

Placenta cGMP level was determined in placental homogenates using ELISA kit from Elabscience (Wuhan, China) with a sensitivity of 0.047 pmol/mL, detection range of 0.78−50 pmol/L and the specificity is recognition of cGMP in sample. No significant cross-reactivity or interference between cGMP and analogues was observed and coefficient of variation is <10 %. A procedure based on competitive-ELISA principle was used for standard solution and sample solution. Colour change was read spectrophotometrically at a wavelength of 450 nm. Concentration of cGMP was determined by comparing the optical density of the samples to the standard curve.

2.7.4. Placenta adiponectin

Placenta adiponectin level was determined in placental homogenates using ELISA kit from Elabscience (Wuhan, China) with a sensitivity of 28.13 pg/mL, detection range of 46.88–3000 pg/mL and the specificity is recognition of rat ADP/Acrp30 in samples. No significant cross-reactivity or interference between ADP/Acrp30 and analogues was observed and coefficient of variation is <10 %. A procedure based on Sandwich-ELISA principle was used for standard solution and sample solution. Colour change was read spectrophotometrically at a wavelength of 450 nm. Concentration of ADP/Acrp30 was determined by comparing the optical density of the samples to the standard curve.

2.7.5. Markers of oxidative capacity and mitochondrial function assayed by standard colorimetric kits

Lactate dehydrogenase (LDH) Assay Kit (Colorimetric) quantifies LDH activity in a variety of biological samples. In this assay LDH reduces NAD to NADH, which then interacts with a specific probe to produce a color (λmax =450 nm). L – Lactate Assay Kit (Colorimetric) is a kit where lactate is oxidized by lactate dehydrogenase to generate a product which interacts with a probe to produce colour (λmax =450 nm). The Aconitase Activity Assay kit provides a simple and direct procedure for measuring Aconitase activity in a variety of samples. Aconitase activity is determined in a coupled enzyme reaction in which citrate is converted to isocitrate by aconitase. This results in a colorimetric (450 nm) product proportional to the enzymatic activity present. One unit of aconitase is the amount of enzyme that will isomerize 1.0 μmole of citrate from isocitrate per minute at pH 7.4 at 25 °C.

2.7.6. Placenta lipids

Free fatty acid Assay Kit (Colorimetric) provides a convenient, sensitive enzyme-based method for detecting the long-chain free fatty acids in various biological samples. In this assay, fatty acids are converted to their CoA derivatives, which are subsequently oxidized with the concomitant generation of color (at λ = 570 nm). The estimation of Triglyceride (TG) and Total cholesterol (TC) were done using the non-enzymatic colorimetric assay kit (at λ = 570 nm)

2.7.7. Placenta lipid peroxidation

Malondialdehyde (MDA) placenta homogenates levels of thiobarbituric acid reactive substances (TBARS), product of malondialdehyde (MDA), were assayed through spectrophotometric method (using kit from Oxford Biomedical Research Inc. (Oxford, UK). The standard, sample and sample blank were incubated for 45 min. Plot a standard curve using the optical density (OD) read at 532 nm (A532 OD) values for each standard versus the MDA concentration for each standard was plotted. The equation of the line can be found using a linear fit method. The sample blank OD (A532) was subtracted from the sample OD (A532) to obtain a net OD (A532). The MDA concentration for each sample was calculated using the net OD (A532)

2.7.8. Markers of tissue injury

Aspartate aminotransferase (AST) was determined Using AST Activity Assay Kit, an amino group is transferred from aspartate to α-ketoglutarate. The products of this reversible transamination reaction are oxaloacetate and glutamate. The glutamate is detected in a reaction that concomitantly converts a nearly colorless probe to color (λmax =450 nm). Alanine transaminase (ALT) Activity was determined using

Assay Kit (Colorimetric), a kit where ALT catalyzes the transfer of an amino group from alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate. The pyruvate is detected in a reaction that concomitantly converts a nearly colorless probe to both color (λmax = 570 nm).

2.7.9. Nrf2-dependent antioxidant markers

Superoxide dismutase-1 (SOD-1), superoxide dismutase-2 (SOD-2), catalase were determined by standard colorimetric methods. Superoxide Dismutase (SOD) Activity Kit is designed to quantitatively measure SOD activity. The assay measures all types of SOD activity, including Cu/Zn, Mn, and FeSOD types. A bovine erythrocyte SOD calibrator is provided to generate a calibrator curve for the assay and all samples should be read off of the calibrator curve. Samples are diluted in specially colored Sample Diluent and added to the wells. The Substrate is added followed by Xanthine Oxidase Reagent and incubated at room temperature for 20 min. The Xanthine Oxidase generates superoxide in the presence of oxygen, which converts a colorless substrate in the Detection Reagent into a yellow colored product. The colored product is read at 450 nm. Increasing levels of SOD in the samples causes a decrease in superoxide concentration and a reduction in yellow product. Catalase reacts with a known quantity of H2O2. The reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase (HRP), remaining H_2O_2 reacts with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample. The colour is read at 510 nm.

2.7.10. Placenta PDE5 activity and nitric oxide levels

Nitric oxide (NO) and PDE5 activity were measured by non-enzymatic colorimetric assay obtained from Oxford Biomedical Research Inc (USA). NO was assayed spectrophotometrically by measuring the accumulation of its stable degradation products, nitrate and nitrite. Quantification of nitrite using a microplate reader at 540 nm within 20 min of the reaction and colour formation was carried out. Placenta PDE5 activity was assayed on the basis of cleavage of cAMP or cGMP by a cyclic nucleotide phosphodiesterase to form 5'-AMP or 5'-GMP respectively. The 5'-nucleotide released is further cleaved into the nucleoside and phosphate by the enzyme 5'- nucleotidase. The phosphate released due to enzymatic cleavage is quantified in the presence of a highly sensitive phosphate detection solution (green assay reagent) which gives a green colour that is measured at 620 nm wavelength.

2.8. Statistical analysis

Data were expressed as means ± SEM. Statistical analysis was performed using One-way analysis of variance (ANOVA) and Bonferroni's Post hoc to identify the significance of pair wise comparison of mean values among the groups with statistical package for social sciences. Statistically significant differences were accepted at 95 % confidence interval ($p < 0.05$).

2.9. Strength and limitations

In the present study, testosterone levels in the circulation and placenta were evaluated and associated with systemic glucoregulation, placental adiponectin level, placental lipid accumulation, PDE5 activity, nitric oxide level and antioxidant levels in control, testosterone-treated and sildenafil + testosterone-treated animals. However, a limitation of the study is that adiponectin signaling via the adiponectin receptor (s) and transduction mechanism was not evaluated.

3. Results

3.1. Effect of sildenafil on placental and plasma testosterone level in testosterone-exposed pregnant animals

Testosterone level was not altered across the groups in the placenta despite 4-fold increase in plasma testosterone compared with control in testosterone-exposed pregnant animals without sildenafil and 6-fold of mean levels of testosterone in animals exposed to gestational testosterone with sildenafil (Fig. 1). This result shows that sildenafil potentiates plasma testosterone levels during testosterone exposure in pregnant rats but the placenta resists testosterone accumulation and may prevent androgenization of fetus in rats.

3.2. Effect of sildenafil on glucose tolerance in testosterone-exposed pregnant animals

Testosterone exposure during pregnancy increased blood glucose especially at one hour post-load period in the oral glucose tolerance test (OGTT) compared with control. The area under the curve of OGTT was increased by testosterone exposure to pregnant rats compared with control. The triglyceride-glucose index and triglyceride-high density lipoprotein ratio were also increased by testosterone exposure to pregnant rats when compared with control group (Fig. 2). Gestational excess testosterone caused overt maternal glucose deregulation. Sildenafil treatment to testosterone-exposed pregnant rats reduced one hour post-load glycemia, area under the curve of OGTT and triglyceride-glucose index compared with testosterone exposure without sildenafil but were not affected compared with control. The triglyceride-high density lipoprotein ratio increased in sildenafil-treated testosterone-exposed pregnant animals compared with control but was not altered compared with gestational testosterone exposure without sildenafil. Gestational excess testosterone impacts on maternal tissues including placenta and indirectly fetus can be linked to glucose deregulation and sildenafil has protective potential as indicated in Fig. 2.

3.3. Effect of sildenafil on placental mitochondrial function and lipid accumulation in testosterone-exposed pregnant animals

Gestational testosterone exposure did not alter placental free fatty acid compared with control (Fig. 3C) but it increased placental lactate dehydrogenase and lactate (Fig. 3A and B), triglyceride (Fig. 3E), total cholesterol (Fig. 3F) and reduced aconitase activity compared with control. Sildenafil treatment during testosterone exposure increased placental lactate dehydrogenase, lactate and aconitase compared with both control and testosterone exposure without sildenafil and both triglyceride and total cholesterol compared with control alone. Also, sildenafil reduced placental free fatty acid compared with gestational testosterone exposure without sildenafil (Fig. 3D). Excess gestational testosterone induced placental lipid accumulation which is consistent with the systemic glucose dysregulation but sildenafil did not avert placental lipid accumulation in testosterone-exposed pregnant rats.

3.4. Effect of sildenafil on placental nuclear factor erythroid 2-related factor 2-dependent antioxidants in testosterone-exposed pregnant animals

Testosterone exposure during late pregnancy reduced placental nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor responsible for expression of antioxidants molecules by approximately 0.6 of the control group level and reduced catalase, a cytosolic antioxidant compared with control (Fig. 4A) but did not affect placental Zn/Cu/superoxide dismutase and Mn/superoxide dismutase compared with control (Fig. 4B and C). Treatment with sildenafil reduced placental Nrf2 and Zn/Cu/superoxide dismutase compared with control but did not alter them compared with testosterone exposure without sildenafil. Sildenafil treatment did not affect placental Mn/superoxide dismutase

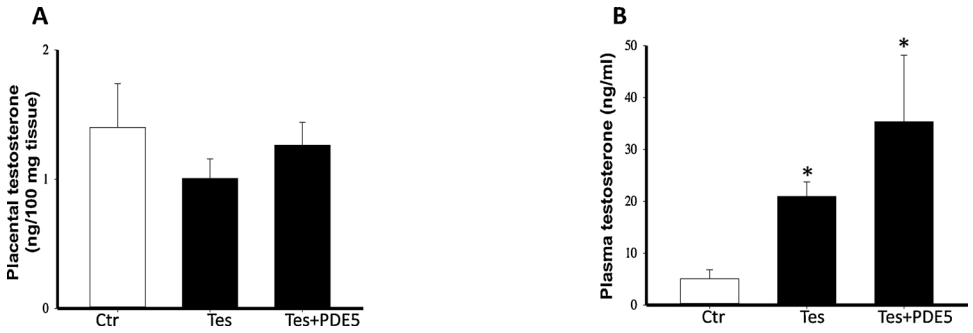

Fig. 1. Effect of Sildenafil on placental and plasma testosterone level in testosterone-exposed pregnant Wistar rats. Sildenafil did not affect placental testosterone (A) and plasma testosterone (B). Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

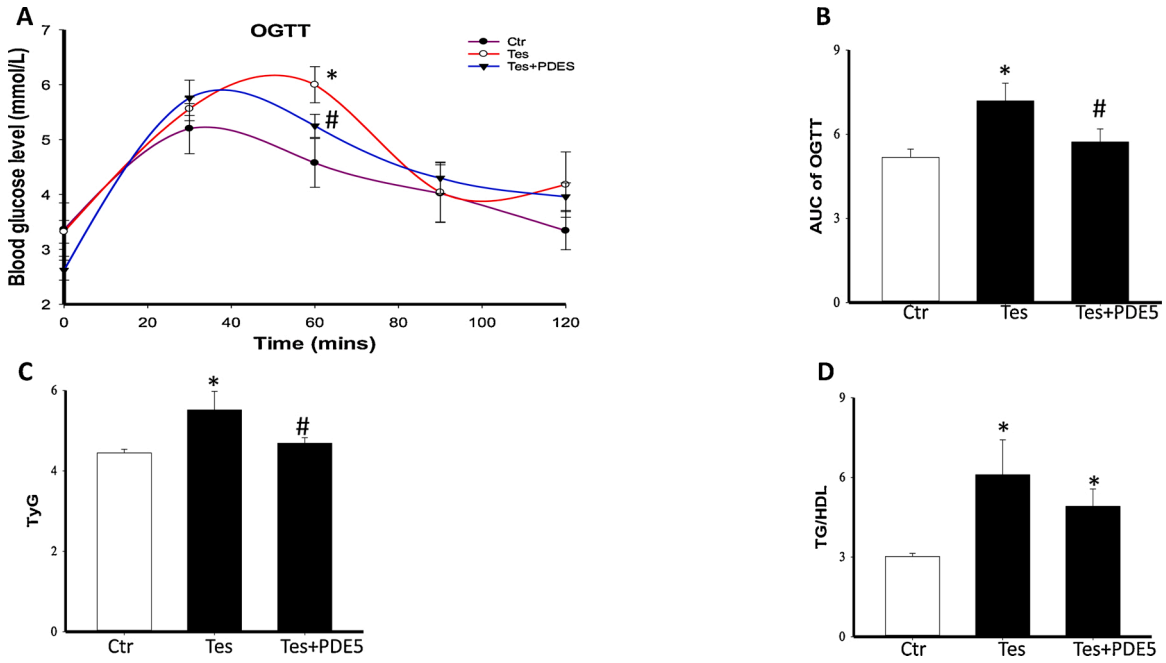

Fig. 2. Effect of sildenafil on glucose tolerance in testosterone-exposed pregnant Wistar rats. Sildenafil restored glucose tolerance (A), reduced area under the curve of oral glucose tolerance test; AUC of OGTT (B), reduced triglyceride-glucose index; TyG (C) and did not affect triglyceride-high density lipoprotein ratio; TG/HDL (D). Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

compared with both control and testosterone exposure without sildenafil but increased catalase compared with both control and testosterone exposure without sildenafil (Fig. 4D). Testosterone had significant ameliorating effect on placental Nrf2 in pregnant rats. This effect was not averted by sildenafil. The only reason here is that sildenafil did not also avert circulating testosterone but increased even further. Circulating testosterone might have diminishing effects on placental Nrf2.

3.5. *Effect of sildenafil on placental adiponectin in testosterone-exposed pregnant animals*

Testosterone exposure to pregnant rats reduced placental adiponectin compared with control. Sildenafil treatment during testosterone exposure increased placental adiponectin compared with both control and pregnant animals exposed to testosterone without sildenafil (Fig. 5). Adiponectin alteration by testosterone may be significantly related to placental functions and fetal changes since improvement of placental adiponectin by sildenafil was associated with improved fetal outcome.

3.6. *Effect of sildenafil on placental phosphodiesterase-5 activity, nitric oxide and cyclic guanine monophosphate in testosterone-exposed pregnant animals*

Testosterone exposure during pregnancy did not affect placental phosphodiesterase-5 (PDE5) activity, nitric oxide and cyclic guanine monophosphate levels compared with control (Fig. 6). Sildenafil treatment during gestational testosterone exposure increased placental PDE5 activity compared with both control and gestational testosterone exposure without sildenafil treatment but reduced placental nitric oxide compared with control (Fig. 6A and B).

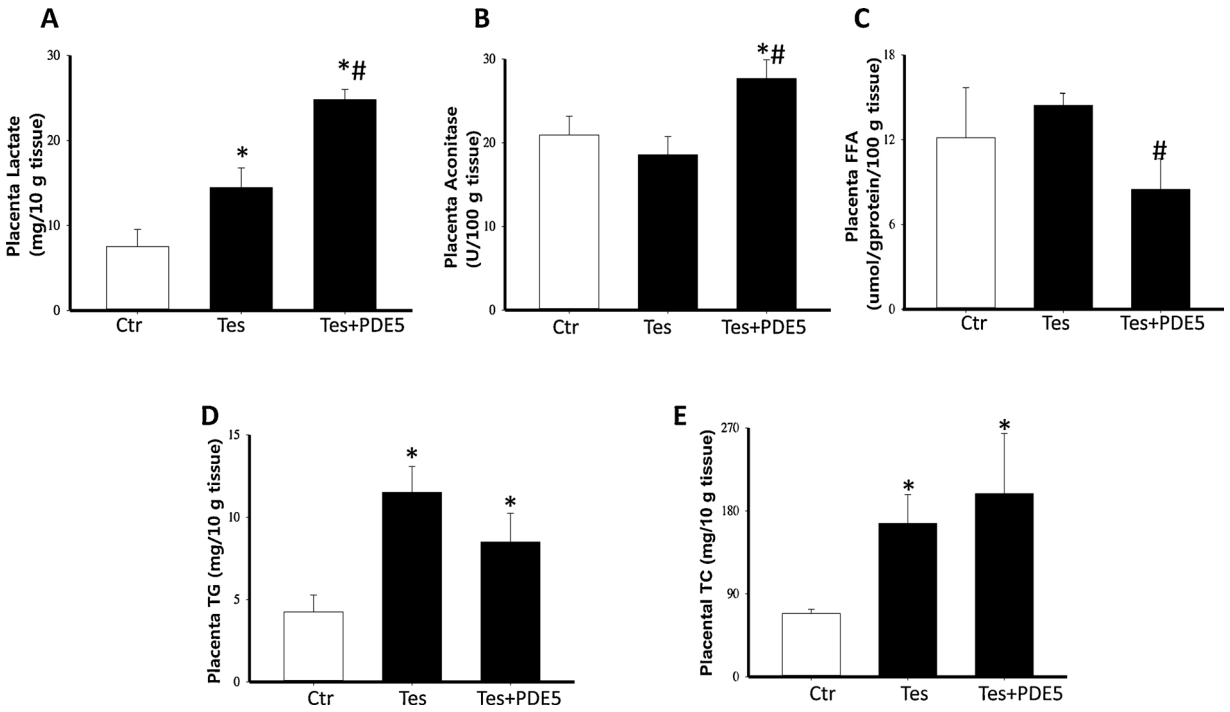

Fig. 3. Effect of sildenafil on placental mitochondrial function and lipid accumulation in testosterone-exposed pregnant Wistar rats. Sildenafil increased placental lactate dehydrogenase (A), lactate (B), aconitase (C), reduced free fatty acid; FFA (D), did not affect placental triglyceride; TG (E) and total cholesterol; TC (F). Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

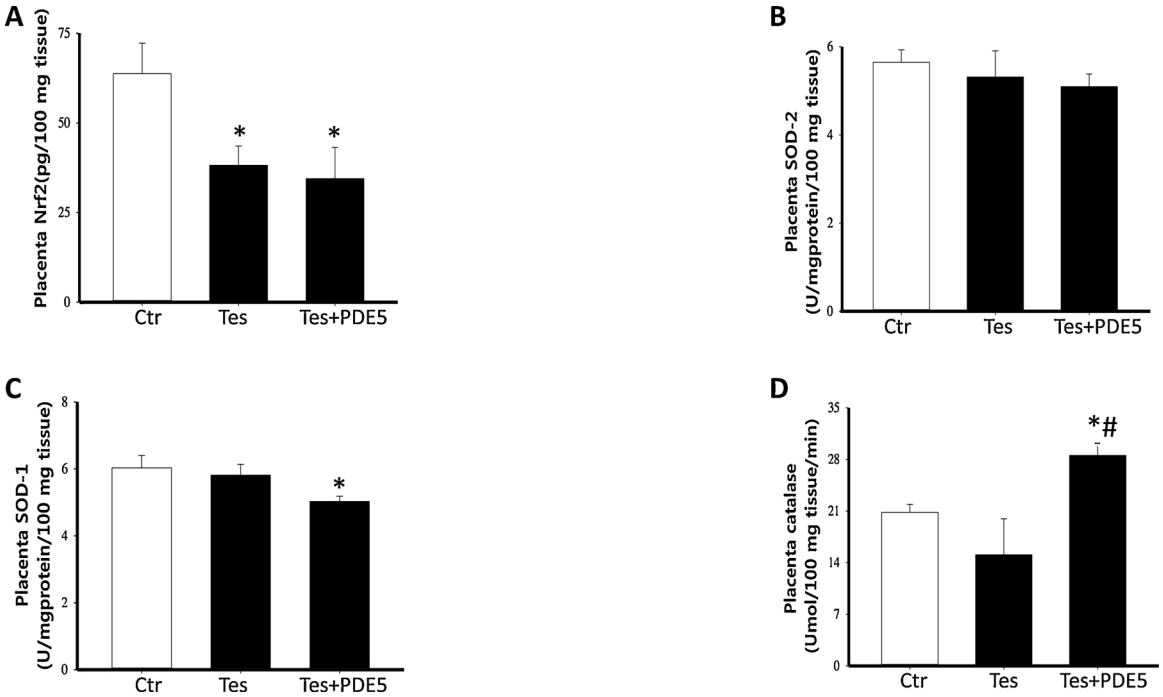

Fig. 4. Effect of sildenafil on placental nuclear factor erythroid 2-related factor 2-dependent antioxidants in testosterone-exposed pregnant Wistar rats. Sildenafil did not affect placental nuclear factor erythroid 2-related factor 2; Nrf2 (A), Mn/superoxide dismutase; SOD-2 (B), Zn/Cu/ superoxide dismutase; SOD-1(C)but increased catalase (D). Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

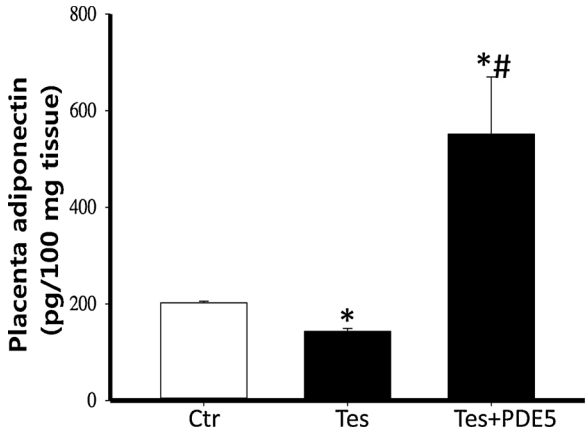

Fig. 5. Effect of sildenafil on placental adiponectin in testosterone-exposed pregnant Wistar rats. Sildenafil increased placental adiponectin. Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

3.7. Effect of sildenafil on placental lipid peroxidation and markers of tissue injury in testosterone-exposed pregnant animals

Testosterone increased placental malondialdehyde (MDA) and alanine amino transferase (ALT) but did not affect aspartate transaminase compared with control in pregnant rats (Fig. 7). Sildenafil treatment to testostosterone-exposed pregnant rats increased MDA compared with both control and pregnant animals exposed to testosterone without sildenafil (Fig. 7A) but did not affect ALT (Fig. 7B) and AST (Fig. 7C).

3.8. Effect of sildenafil on fetal and placental weights in testosterone-exposed pregnant animals

Testosterone exposure during pregnancy reduced fetal and placental weight compared with control (Fig. 8A and C). Testosterone exposure during pregnancy did not affect fetal number compared with control but reduced placental efficiency (Fig. 8B and D). Sildenafil treatment during gestational testosterone exposure increased fetal and placental weight compared with testosterone exposure without sildenafil but not control (Fig. 8A and C). Sildenafil treatment during gestational testosterone exposure increased fetal number and increased placental efficiency compared with testosterone exposure without sildenafil but remained lower than control (Fig. 8D).

3.9. Effect of sildenafil on fetal gross appearance in testosterone-exposed pregnant animals

Testosterone exposure during pregnancy led to smaller looking and

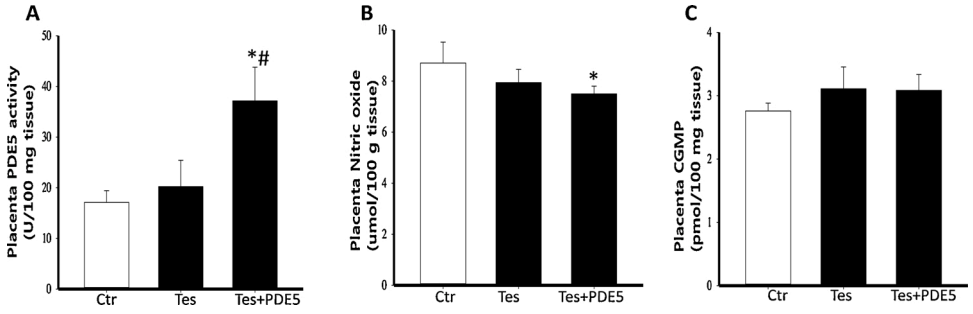

Fig. 6. Effect of sildenafil on placental phosphodiesterase-5 activity, nitric oxide and cyclic guanine monophosphate in testosterone-exposed pregnant Wistar rats. Sildenafil increased placental phosphodiesterase 5 activity; PDE5 activity (A), did not affect placental nitric oxide (B) and cyclic guanine monophosphate; cGMP (C). Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

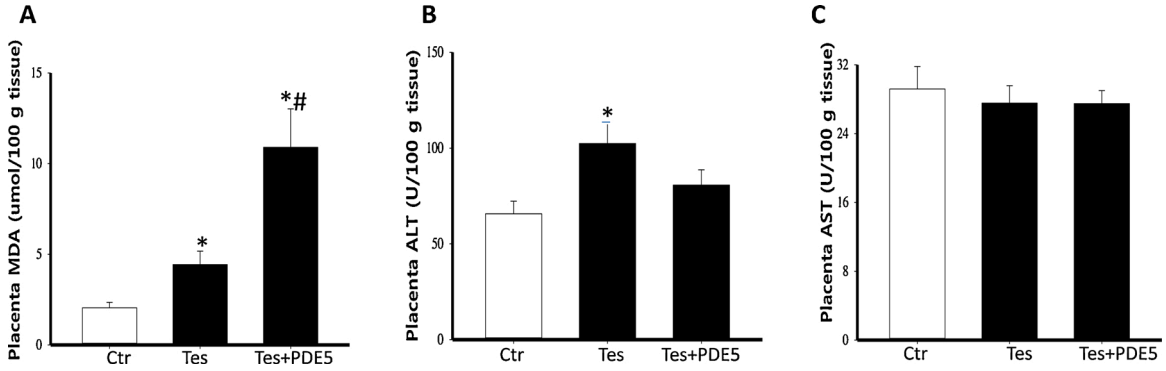

Fig. 7. Effect of sildenafil on placental lipid peroxidation and markers of tissue injury in testosterone-exposed pregnant Wistar rats. Sildenafil increased placental malondialdehyde; MDA (A), did not affect placental alanine transferase ALT (B) and aspartate transaminase; AST (C). Gestational testosterone exposure and sildenafil treatment were carried out in days 14-19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

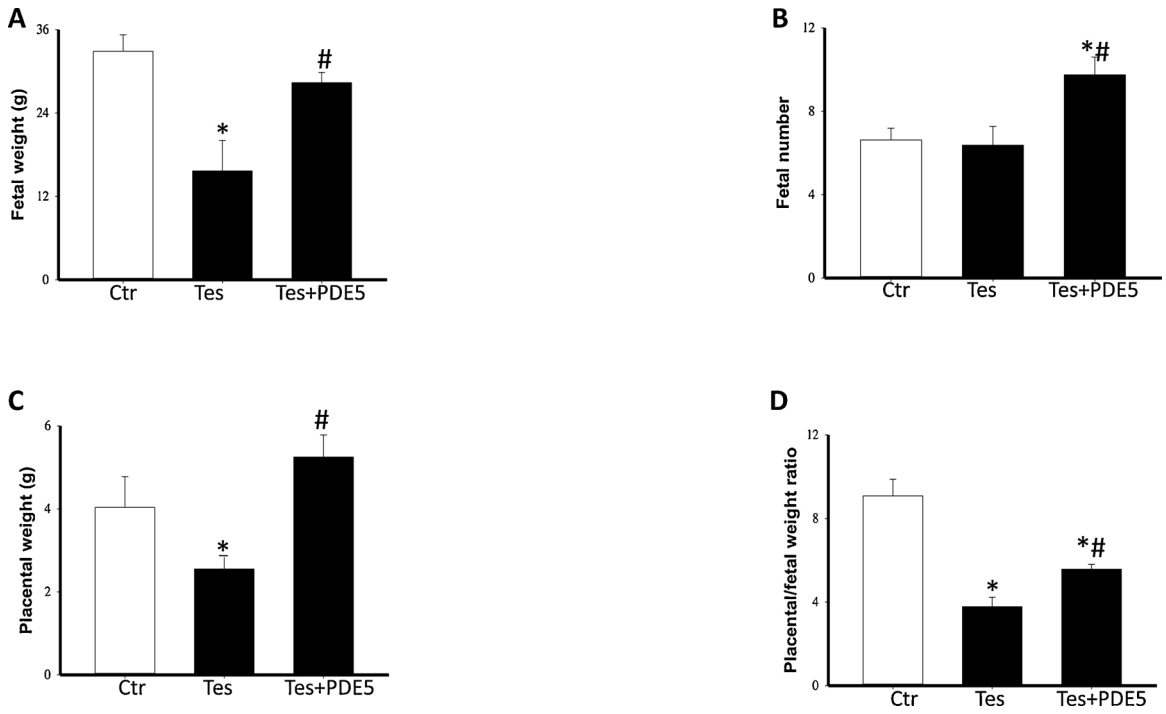

Fig. 8. Effect of sildenafil on fetal and placental outcome in testosterone-exposed pregnant Wistar rats. Sildenafil increased average fetal weight per group (A), Fetal number (B), placental weight per group (C), placental weight per fetus (D), did not affect fetal weight fetus (E) and reduced placental-fetal weight ratio (F). Gestational testosterone exposure and sildenafil treatment were carried out in days 14–19 of gestation. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure. Data were presented as mean ± standard error of mean and $P < 0.05$ was taken as statistically significant. *$P < 0.05$ vs. Ctr; #$P < 0.05$ vs Tes. Data were analyzed by one-way ANOVA and Bonferroni Post-hoc test.

fragile fetuses indicating overt intrauterine growth restriction and malnutrition whereas, silldenafil treatment to testosterone-exposed pregnant rats led to bigger and stronger looking fetuses (Fig. 9).

4. Discussion

The findings of the present study reinstated the observation in previous human and animal studies that gestational hyperandrogenism could result in maternal metabolic disturbances and poor fetal outcome [14,15]. However, this study went further to investigate the effect of PDE5i, sildenafil on fetal weight, glucose tolerance, placental adiponectin/Nrf2 signaling, aconitase activity and lipid accumulation in testosterone-exposed pregnant rats. It was found here that late gestational testosterone exposure in rats impaired glucose tolerance, reduced fetal and placental weight and caused placental inefficiency. However, the finding was associated with reduced placental adiponectin, Nrf2 and catalase independent of placental testosterone. Nevertheless, sildenafil alleviated glucose intolerance and averted placental and fetal development.

Gestational exposure to subcutaneously administered testosterone propionate (3 mg/kg) from gestational day 14–19 increased circulating testosterone by approximately 4-folds (Fig. 1A) but did not significantly affect placental testosterone level (Fig. 1B). This outcome of placental testosterone attenuation relative to circulating testosterone level implies

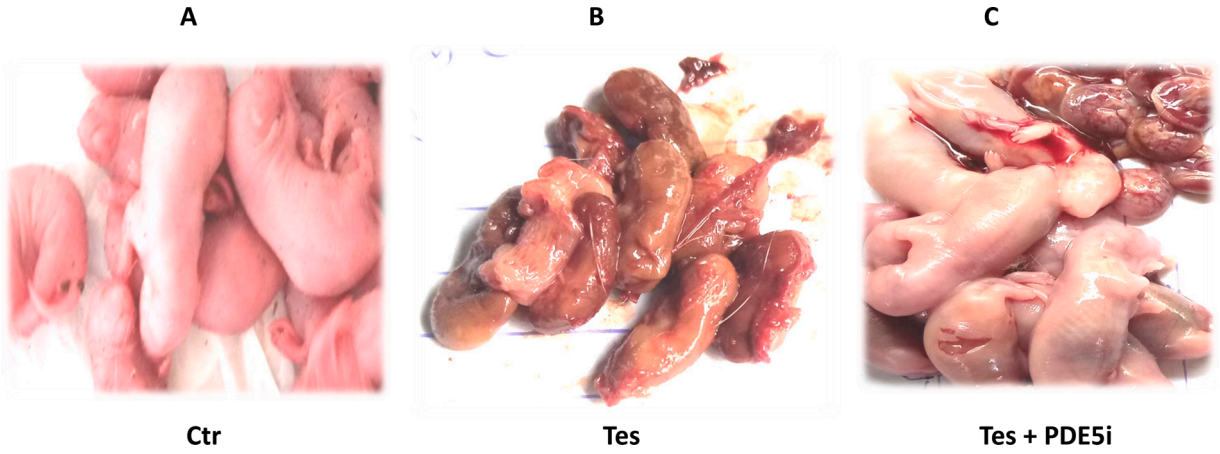

Fig. 9. Diagrams showing the effects of vehicle (A), testosterone (B) and testosterone with sildenafil (C) on fetal outcome. Ctr: animals treated with olive oil subcutaneously and distilled water orally; Tes: animals treated with testosterone propionate subcutaneously; Tes + PDE5: animals treated with sildenafil during testosterone exposure.

that although testosterone crosses the placental membrane, increased activity of placental steroidogenic enzymes such as Δ4 -reductase and 17b-hydroxysteroid dehydrogenase but not aromatase converts testosterone to less active androgens [36] in rats since rat placenta does not express aromatase [37]. In humans however, studies have shown that placental aromatase activity suppresses gestational hyperandrogenism [38], contributes to steroidogenesis and prevents excess fetal androgenization [39]. Gestational maternal and fetal exposure to excess free testosterone therefore occurs in PCOS or preeclampsia in which placental defensive mechanism is suppressed or overwhelmed [40,41]. The present study did not investigate fetal androgenization but the poor fetal and placental phenotypes observed (Fig. 9) with testosterone reflects that despite low placental testosterone, increased circulating testosterone might elicit its effect on placental and fetal development by interacting with a membrane receptor such as the androgen sensitive G-protein coupled receptor. This can lead to inracellular metabolic modifications that might shortchange the fetus of basic supplies.

Testosterone-induced alteration of glucoregulation in pregnant animals has been identified in studies from our laboratory [42,43]. The present study accentuates the existing knowledge as gestational testosterone exposure impaired glucose tolerance by OGTT and AUC of OGTT [Fig. 2A, B]. Besides, TyG and TG/HDL were also elevated. The TyG has been shown to indicate metabolic syndrome better than other indicators like fasting glucose and triglycerides [44]. Metabolic syndrome is usually related to systemic insulin resistance (IR) which is the major cause of glucose intolerance and leads to ectopic lipid accumulation. The placenta provides maternal-fetal exchange interface and carries out metabolic modifications which directly impacts developing fetus. As a maternal tissue, the placenta can receive the deleterious impact of overt dysmetabolism like ectopic lipid accumulation. The present study showed that gestational testosterone exposure increased placental TG and TC but did not significantly affect FFA (Fig. 3). Lipid accumulation affects fetal growth by altering glucose utility and engenders pathologies like preeclampsia [45]. Aside inhibiting glucose utility, fatty acid intermediates which stimulate the production of inflammatory cytokines and oxidants damage molecules responsible for nutrient utility and transport. Here, excess placental lipid accumulation (Fig. 3D) was accompanied by elevated lipid peroxidation and alanine amino transferase which are markers of oxidative tissue damage (Fig. 7A). Also, the placental elevated lactate production and aconitase observed here indicate lactate dehydrogenase complex inhibition and mitochondrial dysfunction respectively (Fig. 3A). Nevertheless, reduced aconitase activity positively feeds back on lipid accumulation through citrate build up in the cytosol leading to cyclically consistent mitochondrial dysfunction and deficient nutrient utility which might result in poor placental and fetal development.

The aim of this study was to investigate the link between the maternal testosterone-induced placental derangement and pregnancy outcome. The function of the placenta is dependent on transport structures which can be damaged by overt oxidative stress. Whereas, the ubiquitous master antioxidant/anti-inflammatory intracellular activator is the Nrf2. Testosterone reduced placenta Nrf2 in this study and placental Nrf2-dependent antioxidant status here showed that despite reduction in Nrf2 and catalase, SOD-1 and 2 were not significantly affected by gestational testosterone exposure (Fig. 4). Testosterone therefore might have stimulated inhibition of Nrf2 by elevating expression of keap1 genes that increases Nrf2 ubiquitylation without directly affecting Nrf2-signaling. Also the effect of testosterone on Nrf2 could not be accounted for by intramural testosterone level in the placenta of testosterone-exposed pregnant rats but could be explained by the fact that despite resistance of testosterone accumulation in the placenta, there is plausibility that testosterone interaction with G-protein coupled membrane receptors can cause intracellular signaling that diminishes Nrf2 molecule. This has to be specified by further studies. Catalase converts peroxides (H_20_2) to water and deficiency of this enzyme will elevate intracellular H_20_2 and engender tissue damage via peroxidation. The reduction of catalase in the placenta induced by gestational testosterone exposure is consistent with the increased lipid peroxidation and aconitase deactivation observed here because aconitase is oxidant-sensitive. Reduced aconitase activity is associated with lipid accumulation via cytosolic citrate accumulation leading to mitochondrial dysfunction and increased lactate production.

In the present study, placental adiponectin an anti-inflammatory adipokine was also investigated and it was found that gestational testosterone exposure reduced placental adiponectin level (Fig. 7). Adiponectin is a known anti-inflammatory adipokine and its suppression can contribute to complication of metabolic syndrome. The expression of adiponectin in the placenta is a promising therapeutic target that has not received deserving attention. However, a study had linked reduced placental adiponectin expression to poor placental development in gestational diabetes mellitus [46]. In contrast, other studies have shown that adiponectin at late pregnancy might reduce nutrient supply to fetus [47]. The outcome of the present study shows that placental adiponectin reduction by testosterone is associated with reduced placental weight and fetal weight. Also, placenta adiponectin here might be related to cord adiponectin which has been positively correlated with birth weight [48]. Adiponectin reduction in the placenta will fail to adequately stimulate AMPK signaling leading to attenuated lipid oxidation and consequent lipid accumulation in testosterone-induced glucose intolerance as observed in this study. Lipid accumulation in the placenta has been associated with abnormal placental development [49] as observed in this study. Poor placental development will shortchange the fetus whether through compromised blood flow or nutrient supply. The placental PDE5/cGMP/Nitric oxide pathway was investigated to evaluate the impact of gestational testosterone on nitric oxide-related blood flow in the feto-placental region to assess the level to which vasodilation might affect fetal growth in gestational hyperandrogenemic milieu. Surprisingly, this pathway was not affected. This suggests that blood flow was not significantly compromised as the vasodilator nitric oxide remained unchanged. It is therefore noteworthy that rather than the blood flow, it is the content of the blood (reduced wanted materials and increased unwanted materials) that might well define the impact of testosterone on fetal outcome.

The drug sildenafil was administered to testosterone-exposed pregnant rats in this study to investigate therapeutic and preventive effect on specific placental biochemistry and fetal outcome. However, it was found that sildenafil increased circulating testosterone compared with both control and testosterone-exposure without sildenafil. A study had shown that sildenafil increases testosterone secretion in male testes by increasing the conversion of progesterone to testosterone [50]. Thus, the use of sildenafil in hyperandrogenic milieu should be with caution. Nevertheless, sildenafil improved glucose tolerance (Fig. 2) in this study despite elevated circulating teststerone. Sildenafil is known to enhance insulin secretion [51] which might contribute to the improved glucose tolerance realized here. Improving insulin secretion may delay the deleterious TG accumulation in the placenta which was accentuated by significantly attenuated FFA and slightly attenuated triglyceride in the placenta. Although lactate production remained high with sildenafil in testosterone-exposed pregnant rats, catalase and aconitase activity was significantly improved. Despite aconitase and catalase improvement there was no change in placenta Nrf2 and nitric oxide compared with testosterone exposure without sildenafil. The unaltered Nrf2 and nitric oxide with sildenafil compared with testosterone exposure without sildenafil might explain why lipid peroxidation was not reversed with the same treatment (Fig. 8A). Nrf2 promotes development of placenta and its functions [52]. Hence, in the present study, it is tempting to speculate that reduced Nrf2 plays significant role in the poor placental growth observed in gestational testosterone-exposed animals. Nevertheless, lack of reverse of placental Nrf2 by sildenafil despite placental weight recovery could imply that Nrf2 reduction does not play a major role in the pathogenesis of testosterone-induced poor placental and fetal development. The outcome here also shows that catalase activity is not sufficient

to prevent lipid peroxidation in cells especially for the fact that Nrf2 can on its own avert inflammation and prevent oxidative stress without transcription functions. However, sildenafil augmented placental adiponectin and improved fetal and placental growth in gestational testosterone-exposed animals. Adiponectin has been positively correlated with fetal weight [20] and appears in this study to be significantly involved in both placental growth and fetal development. We propose that adiponectin through a lipid regulatory-dependent mechanism modulates growth and functions in the placenta and fetus. Nevertheless, there is no evidence here that adiponectin modulated Nrf2 levels or signaling in this study.

5. Conclusion

In conclusion, gestational high testosterone-induced poor placental and fetal outcome is characterized by glucose intolerance, reduced placental adiponectin, Nrf2, aconitase, increased lipid accumulation, lipid peroxidation and lactate production but without effect on nitric oxide levels. However, sildenafil treatment to testosterone-exposed pregnant animals reversed placental and fetal outcome by augmenting placental adiponectin and preventing excess triglyceride accumulation with accompanied improved glucose tolerance regardless of reduced placental Nrf2 and high circulating testosterone. The use of sildenafil in preventing IUGR can cause improvement in fetal outcome through a placental adiponectin enhancing mechanism independent of Nrf2 signaling.

Author statement

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Conflict of interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

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