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Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague–Dawley rats

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ABSTRACT

Objectives: This study aimed to investigate the effects of sildenafil citrate on various fetal and physiological parameters, including fetal mortality, number of pups, placental weights and micro-albuminuria in pregnant, L-NAME treated Sprague–Dawley rats.

Study design: Twenty-four pregnant female Sprague–Dawley rats were divided into 3 groups ($n = 8$). In the L-NAME treated group (PRE), L-NAME (0.3 g/l, drinking water) was used to induce pre-eclampsia-like symptoms on day 1 of the experiment. The experimental group (SCT) also received L-NAME (0.3 g/l, drinking water) on day 1 of the experiment. However, sildenafil citrate (10 mg/kg, s.c., daily) was administered as the test compound from day 7 until day 19. The experimental control (CON) did not receive either L-NAME or sildenafil citrate. L-NAME administration was discontinued in both the PRE and the SCT groups on day 19 of the experiment and the animals were given access to normal drinking water *ad libitum*. All the animals were sacrificed on day 20, at which time a laparotomy was performed and the various fetal parameters measured. On day 0 and day 20, blood pressure measurements were recorded non-invasively and protein estimations in 24 h urine samples were conducted.

Results: Sildenafil citrate decreased fetal mortality and protein excretion and further demonstrated a trend toward increasing birth and placental weights in pregnant, L-NAME treated, Sprague–Dawley rats. In addition, sildenafil citrate administration ameliorated the amplification of the L-NAME induced hypertension in the SCT group.

Conclusion: We speculate that sildenafil citrate by potentiating the effects of nitric oxide *in vivo* improves uterine artery blood flow resulting in improved fetal outcomes in pregnant, L-NAME treated, Sprague–Dawley rats.

1. Introduction

Pre-eclampsia/eclampsia syndrome is defined as a multi-system disorder, characterized by the abrupt onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive, non-proteinuric woman [1]. It is a major cause of both maternal and fetal morbidity and mortality [1,3]. The incidence is reported to be between 2 and 7% in well resourced countries and is up to three times greater in under resourced countries [2,3].

Recent insights in the understanding of the pathophysiology of pre-eclampsia indicate that it is regarded as a two-stage disorder [4]. The first stage is one of vascular maladaptation in the placental bed due to failure of the uterine spiral arteries to undergo complete remodelling into wide bore channels, an important vascular modification in normal pregnancies [4–8]. It has been suggested that this vascular maladaptation is associated with a marked reduction in blood flow to the placenta. The second stage is one in which the reduced blood perfusion induces a hypoxic state resulting in the release of a variety of substances including trophoblastic debris, necrotic tissue and excess secretion of anti-angiogenic factors viz. soluble fms-like tyrosine kinase 1 (sFlt-1); soluble endoglin (sEng) and reduced secretion of angiogenic factors; vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which affect virtually every major organ system by causing endothelial dysfunction and systemic vaso-spasm [9]. If left undiagnosed or untreated, pre-eclampsia results in major complications to the mother and baby [10]. The only known cure at present is delivery of the baby and placenta [1,10].

Nitric oxide (NO) is a potent vasodilator that is synthesized from the amino acid L-arginine (L-Arg), by a family of isoenzymes called nitric oxide synthase (NOS) [11]. It has been suggested that

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diminished NO activity may be involved in the pathophysiology of pre-eclampsia. Studies on the levels of endothelial NOS (eNOS) have been reported to be normal in placental villous tissue of pre-eclamptic women [12]. However, levels of the NO intracellular second messenger, cGMP, are shown to be decreased in the placental circulation of pre-eclamptic women. Therefore, it is likely that there is a reduced activity or half-life of NO [12]. A probable mechanism for this reduced NO activity is that NO is rapidly degraded to perooxynitrite ($OONO^-$) by interacting with reactive oxygen species (ROS), especially the superoxide anion (O_2^-) which has been shown to be present in abundance in the pre-eclamptic placenta [13]. Interestingly, NOS has been shown to not only synthesize NO but also facilitate O_2^- production [14]. Both of these biochemical mechanisms are tightly regulated by L-Arg, where sufficient levels of L-Arg have been shown to generate NO only and depletion of L-Arg causes NOS to produce NO and O_2^- [15,16]. These findings have later been confirmed by Noris et al., who demonstrated that L-Arg levels are lower in pre-eclamptic villous tissue in comparison to normal tissue [17]. Subsequently the chronic administration of nitro-L-Arg-methyl ester (L-NAME), a NOS inhibitor, has been shown to induce a pre-eclampsia-like syndrome in pregnant rats, i.e. sustained hypertension, proteinuria and intrauterine growth restriction (IUGR) [7,18].

Since NO may be involved in the pathophysiology of pre-eclampsia, we sought to investigate a possible intervention with an intracellular NO system. Sildenafil citrate (Viagra™), a specific type-5 phosphodiesterase (PDE) inhibitor has been shown to potentiate the effects of NO *in vivo* [19]. Furthermore, it enhances vasodilation and improves the endothelial function of myometrial vessels in pregnancies complicated by IUGR [20]. Other researchers have also shown sildenafil citrate (SC) to improve uterine artery blood flow and endometrial development in women undergoing *in vitro* fertilization [21] as well as having beneficial effects on fetal and vascular parameters in hypertensive pregnant rats [22].

The role of SC, however, in improving pregnancy outcomes in pre-eclampsia still needs further investigation. In this study, we examined the effect of SC on fetal outcomes in a hypertensive rat model by measuring the number of live pups, placental weights and protein concentration in the urine.

2. Methods

2.1. Animal studies

Ethical permission was obtained from the University of KwaZulu-Natal Animal Ethics Committee. The animals were weaned at 4 weeks of age from sister Sprague–Dawley rat litters and the females were then separated from the males. At 8 weeks of age, 24 weight-matched female rats (180–200 g) were randomly divided into 3 groups as follows: Group 1, control [CON] ($n = 8$); Group 2, L-NAME only treated group [PRE] ($n = 8$) and Group 3, SC and L-NAME treated group [SCT] ($n = 8$).

The animals were maintained under standard laboratory conditions for a further 2 weeks on a 12-h light/dark regime and given access to food and water *ad libitum*. Thus, prior to mating, the estrus cycles of these animals were synchronised and vaginal smears were used to determine the phase of their respective cycles. On their next proestrus phase, animals were subjected to the Whitten effect to maximize the sexual receptivity of the female and increase their chances of positive mating. Hence, females in estrus now approximately 10 weeks of age, were mated overnight. The morning after mating had occurred, each animal was examined for the presence of a vaginal plug or the presence of sperm in a vaginal smear. The presence of a vaginal plug or sperm positivity was taken as day 0 of the 21–23 day gestation period.

2.2. Treatment regimen

From day 1 of the experiment, the pregnant females were paired and housed in polypropylene cages where CON group was given normal drinking water and PRE and SCT groups were given L-NAME (0.3 g/l) [Sigma–Aldrich, USA] in their drinking water to induce the pre-eclampsia-like syndrome. From day 7 of the experiment, each animal was treated via subcutaneous injections, daily, at 09:00 as follows: the CON and PRE groups were given the vehicle only (di-methyl-sulfoxide, 0.3 ml) and the SCT group was treated with the study drug (sildenafil citrate) [Pfizer, UK-92480-10] dissolved in di-methyl-sulfoxide (10 mg/kg, b.w.) until day 19 of the experiment.

L-NAME administration was discontinued in both the PRE and SCT groups on day 19 of the experiment and the animals were given free access to normal drinking water thereafter.

2.3. Blood pressure measurements and urinalysis

The blood pressure of each animal was taken on day 0 and day 20 using the non-invasive tail-cuff method (IITC, Life Science, USA). Each animal was pre-trained for 3 consecutive days prior to the day 0 blood pressure measurement to minimize stress reactions. On day 0 and day 19, each animal was housed in an individual metabolic cage (Techniplast, South Africa) with access to water only *ad libitum*. This allowed for a 24-h urine sample collection. Micro-albumin (MA) levels were detected by PEG enhanced immuno-turbidometry on the Advia 1800 system (Siemens, USA).

2.4. Sample collection

On day 20, each animal was sacrificed as described below. Each animal was anaesthetized with halothane (Fluothane™). Blood samples were obtained by cardiac puncture and separated into serum, plasma and whole blood specimens. Thereafter a laparotomy was performed to expose the uterine horns. The number of developed fetuses and their respective placenta were counted, removed and weighed. The utero-placental tissue, heart, kidney, liver and brain were rapidly removed, sectioned and either snap frozen in liquid nitrogen or suspended in formalin or glutaraldehyde for further investigations (these data will be reported separately).

2.5. Statistical analysis

All data were subjected to one-way ANOVA and/or the Tukey–Kramer Multiple Comparison Test using the GraphPad Instat (v.05) statistical software package. Results are presented as mean $\pm$ standard error of the mean (SEM). A probability value of <0.05, was considered statistically significant.

3. Results

3.1. Blood pressure measurements

The systolic blood pressures (SBP) of each group were compared (Fig. 1). There were no significant differences ($p > 0.05$) in the SBPs between the groups on day 0 of the experiment, but on day 20 significant differences were noted amongst all three groups, i.e. CON (135.54 ± 1.17 mmHg) vs. PRE (162.83 ± 0.83 mmHg) [$p < 0.001$]; CON (135.54 ± 1.17 mmHg) vs. SCT (145.46 ± 1.03 mmHg) [$p < 0.001$] and PRE (162.83 ± 0.83 mmHg) vs. SCT (145.46 ± 1.03 mmHg) [$p < 0.001$]. There was also a significant change in SBPs ($p < 0.001$) from day 0 to day 20 within each group. The percentage change in SBPs from day 0 to day 20 for all 3 groups was calculated as follows: [(SBP on day 20–SBP on day 0)/SBP on day 0] $\times$ 100. The CON group showed a