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Maternal hypertension and feto-placental growth restriction is reversed by sildenafil: Evidence of independent effects of circulating nitric oxide levels

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ABSTRACT

Sildenafil has shown nitric oxide (NO)-independent pleiotropic effects, however the mechanisms involved are unclear. We investigated the protective effects of sildenafil against hypertension in pregnancy and feto-placental growth restriction induced by NO inhibition, and if sodium nitrite-derived NO formation influences sildenafil effects. We evaluated the plasmatic levels of NO metabolites, cyclic guanosine monophosphate (cGMP), oxidative stress and myeloperoxidase, which are involved in endothelial dysfunction during hypertension in pregnancy. Also, we performed in vitro experiments to examine cell viability and NO synthesis in human umbilical vein endothelial cells (HUVECs) cultures incubated with plasma from healthy or hypertensive pregnant rats treated (or not) with both drugs, either alone or in association. Sildenafil blunted hypertension in pregnancy and protected against feto-placental growth restriction induced by NO inhibition and these effects of sildenafil alone were similar to those presented by its association with sodium nitrite. Protective effects of sildenafil were observed even with low plasmatic NO levels and were not followed by increases in cGMP levels. Also, sildenafil, but not sodium nitrite, blunted the increases in myeloperoxidase activity. Both drugs (isolated or in association) presented antioxidant effects. Plasma from hypertensive pregnant rats treated with sildenafil, but not sodium nitrite alone, increased the viability of HUVECs. NO synthesis in HUVECs cultures was increased with plasma from rats treated with both drugs. We conclude that sildenafil effects are not dependent of circulating NO levels in hypertension and feto-placental growth restriction. These findings may reflect a protection against myeloperoxidase and pro-oxidant activation in hypertension in pregnancy.

1. Introduction

Hypertensive disorders of gestation complicate about 5–10% of pregnancies, including gestational hypertension that could progress to preeclampsia (Jim and Karumanchi, 2017; Lo et al., 2013). If untreated, these disorders are major causes of maternal and fetal morbidity and mortality (Uzan et al., 2011). Preeclampsia is also associated with intrauterine fetal growth restriction, accounting for 10–15% of preterm births (Mitani et al., 2009); however, the underlying mechanisms of this disorder are unclear. The initiating event is widely believed to be the impaired spiral artery remodeling that, in turn, leads to a stage of poor placentation with posterior ischemia/hypoxia (Roberts, 2014). Ischemic placenta releases soluble factors into maternal circulation, resulting in the secondary stage of the disorder featured by endothelial dysfunction (Possomato-Vieira and Khalil, 2016).

Physiological blood pressure during pregnancy may rely greatly on the vasodilatory action of nitric oxide (NO) (Leiva et al., 2016). NO also seems to influence the cytotrophoblast invasion and mediates the spiral artery remodeling to allow an adequate supply for the growing fetus (Velicky et al., 2016). In fact, circulating levels of nitrite, a NO metabolite, are increased in normal pregnant women compared to both healthy non-pregnant and preeclamptic women (Cadnapaphornchai et al., 2001). Pregnant rats develop hypertension and feto-placental growth restriction if NO formation is pharmacologically reduced by Nω-Nitro-L-arginine methyl ester (L-NAME), an agent that effectively inhibits endothelial, neuronal and inducible NO synthases (Ramesar et al., 2010).

Sildenafil is clinically used to treat erectile dysfunction (Hatzimouratidis, 2006). The known mechanism of sildenafil's action is the inhibition of phosphodiesterase type 5 (PDE5), which lengthens the NO–cyclic guanosine 3′,5′-monophosphate (cGMP) signaling by preventing the degradation of cGMP (Francis et al., 2010). Hence, based on

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this canonic mechanism of sildenafil in potentiating NO-induced vasodilation, one may consider that as a NO-dependent drug, sildenafil would have no potential to achieve therapeutic goals, considering there may be reduction of NO in preeclampsia or when the endogenous NO synthesis is reduced by L-NAME in pregnant rats (Motta et al., 2015). However, previous studies showed that sildenafil attenuates hypertension and feto-placental growth restriction in L-NAME-treated rats (Nassar et al., 2012; Ramesar et al., 2010) as well as in hypertensive pregnant mice deficient in endothelial NO synthase (Roberts et al., 2016). Together, these preclinical findings suggest that sildenafil effects may not depend of circulating NO levels (Chrysant and Chrysant, 2012). However, mechanistic studies are needed to explain these sildenafil effects and to determine its potential efficacy in hypertensive disorders of gestation complicated by fetal growth restriction (Trapani et al., 2016), even with reduced levels of NO (Sandrim et al., 2008).

The main hypotheses tested in the present study were that sildenafil, independently of NO levels into maternal circulation, attenuates hypertension-in-pregnancy and feto-placental growth restriction and that these effects could be associated with endothelial cells protection against oxidative stress.

2. Materials and methods

2.1. Animals and experimental protocol

Wistar rats (200–250 g) were housed in cages at 22 ± 2 °C on a 12-hr light/dark cycle and given free access to water and rat chow. Each female rat was separately mated overnight. Day 1 of pregnancy was defined as the day when spermatozoa were found in a vaginal smear.

On pregnancy day 14, each pregnant rat mother was first placed into a single cage and randomized to one of the eight treatment groups (n = 8–10 per group): Normal Pregnant (NP), Normal Pregnant + Sildenafil (NP + S), Normal Pregnant + Nitrite (NP + N), Normal Pregnant + Sildenafil + Nitrite (NP + S + N), Hypertensive Pregnancy (HP), Hypertensive Pregnancy + Sildenafil (HP + S), Hypertensive pregnancy + Nitrite (HP + N) and Hypertensive Pregnancy + Sildenafil + Nitrite group (HP + S + N). In hypertensive pregnant groups (HP groups), rats received intraperitoneal (i.p.) injections of L-NAME (Sigma, St. Louis, MO, #5751) 60 mg/kg/daily from 15th – 21st gestational day (Yang et al., 2011). Sildenafil citrate (Pfizer, UK-92480-10) was administered by gavage at a dose of 10 mg/kg/day from 15th – 21st gestational day (Baijnath et al., 2014). Sodium nitrite was administered by gavage at dosage of 15 mg/kg/day (Sigma, St. Louis, MO, #S2252) from 15th – 21st gestational day. The dose of sodium nitrite was chosen with basis on previous studies showing that this dose exerts relevant antihypertensive and antioxidant effects in rats (Gonçalves-Rizzi et al., 2016; Montenegro et al., 2011; Pinheiro et al., 2014, 2015).

Rats were euthanized on gestation-day 21 under overdose of isoflurane followed by exsanguination. Blood samples were collected in lyophilized ethylenediamine tetraacetic acid (EDTA, Vacuntainer Becton-Dickinson, BD, Oxford, UK), immediately centrifuged and plasma was separated and stored at − 80 °C until use for biochemical analysis.

All procedures for animal experimentation were approved by the Ethics Committee, Biosciences Institute of Botucatu, São Paulo State University (Protocol #618/2014), which is complied with international guidelines of the European Community for the use of experimental animals.

2.2. Blood pressure measurements

Systolic blood pressure (mmHg) was measured on gestational day 14 (baseline with absence of gavage or i.p. injections) and days 16, 18 and 20, before drugs administration, using tail-cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF 306). Briefly, all pregnant rats were first acclimated in a quiet room, conditioned and restrained for 5–10 min in a warm box (Insight, Ribeirao Preto, Sao Paulo, Brazil, # EFF307) to the measurements for 3 days before the pregnancy day 14 (these data were discarded) and then the baseline systolic blood pressure was determined as the average of the cuff inflation-deflation 3–6) cycles by a trained operator on pregnancy day 14 (Gonçalves-Rizzi et al., 2015).

2.3. Effects on placenta and fetuses

On gestational day 21, after euthanasia, animals were placed in supine position and cesarean section was performed. The number of viable fetuses, litter size, fetal weight and placental weight were recorded. Viable fetuses were determined as those which showed no macroscopical sign of malformation and could apparently have a normal outcome with the progression of the pregnancy, as previously reported (Ma et al., 2010).

2.4. Determination of myeloperoxidase activity

Myeloperoxidase activity was determined by measuring tetramethylbenzidine (TMB) oxidation in an end-point colorimetric assay. For that, 30 µl of plasma (1:100) were incubated with 20 µl of phosphate buffer and 100 µl of liquid substrate system, composed by TMB (Sigma, St. Louis, MO, USA) and hydrogen peroxide 0.04%, at 37 °C for 10 min, protected from light. After incubation, the reaction was stopped with 100 µl of H_2SO_4 (2 N) and the absorbance at 450 nm with correction to 630 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). The results were expressed in Myeloperoxidase activity (U/L) (Suzuki et al., 1983).

2.5. Measurements of plasma antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC) was performed as previously described (Erel, 2004). Briefly, a standard curve was stablished using 100 µg of Trolox (6-hidroxy-2,5,7,8 - tetramethylchroman-2-carboxylic-acid, Sigma, St. Louis, MO, USA, catalogue# 238813) in 1 ml of sodium acetate buffer (0.4 M, $C_2H_3NaO_2{\cdot}3H_2O$) + glacial acetic acid (0.4 M). Firstly, 20 µl of plasma samples were added to 200 µl of sodium acetate buffer + glacial acetic acid and the absorbance at 660 nm was read with the spectrophotometer (Synergy 4, BIOTEK, Winooski, VT, USA). Secondly, 20 µl of sodium acetate buffer (0.03 M) and glacial acetic acid (0.03 M) + H_2O_2 + ABTS (2,2′-azino-bis-3-ethylbenz-thiazolin-6 sulfonic acid, Sigma A 1888) was added to the samples and incubated for 5 min. Finally, a second spectrophotometer read was performed at 660 nm. The second reading values were subtracted from the values found in the first reading and the antioxidant activity of the sample was expressed as mmol of Trolox equivalent/L.

2.6. Assessment of lipid peroxidation

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid-reactive substances (TBARS) (Perico et al., 2015). In test tubes, 100 µl of distilled water, 50 µl of 8.1% sodium dodecyl sulfate (SDS), 375 µl of 20% acetic acid pH 3.5, and 375 µl of 0.8% 2-thiobarbituric acid (TBA) diluted in 20% acetic acid were added to 100 µl of sample. For the standard curve, the test tubes contained 25 µl of a malondialdehyde solution of known concentration, 175 µl of distilled water, 50 µl of 8.1% sodium dodecyl sulfate, 375 µl of 20% acetic acid pH 3.5, and 375 µl of 0.8% 2-thiobarbituric acid (TBA) diluted in 20% acetic acid. The test tubes were incubated in water bath at 95 °C for 1 h and centrifuged at 1792 g for 10 min. A 200 µl aliquot of each sample was transferred to a 96-well plate. The malondialdehyde formed by the sample reacts with the TBA to produce a colorimetric reaction that was measured using a spectrophotometer (Synergy 4, BIOTEK, Winooski,