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Effect of Sildenafil on Pre-Eclampsia-Like Mouse Model Induced By L-Name

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Contents

N(omega)-nitro-L-arginine methyl ester (L-NAME) decreases the vasodilator effect of nitric oxide (NO) and induces pre-eclampsia in mouse. Sildenafil inhibits the degradation of nitric oxide and increases vasodilation. This study aimed to determine the effects of sildenafil citrate on angiogenesis and oxidative stress at the maternal foetal interface on pre-eclampsia-like mouse model induced by L-NAME. Twenty pregnant mice were divided into four groups: (i) vehicle control; (ii) L-NAME; (iii) sildenafil; (4) L-NAME+sildenafil. L-NAME was administered from day 7 of pregnancy and sildenafil from day 8 until day 16; animals were euthanized on day 17. Placental and foetal sizes and weights were measured; lipid peroxide levels and catalase activity in placental homogenates were determined, and placental vascular endothelia were identified by lectin-histochemistry using BSA-I lectin. Western blot analysis was used to determine VEGF expression in placental homogenates. No changes were seen in placental and foetal development in mice with normal pregnancies treated with sildenafil. Treatments with L-NAME reduced significantly the placental weight and average height and decreased the percentage of the endothelial surface. These alterations may be mediated by the reduction of NO levels in trophoblastic cells, due to the inhibitory effect of L-NAME on nitric oxide synthase (NOS) synthesis. This effect was offset by the treatment with sildenafil, with an increase in the percentage of the endothelial surface. In conclusion, our results indicate that treatment with sildenafil on pre-eclampsia mouse model can be used without adverse effects on the concept and its use in the treatment of pre-eclampsia is promising.

Introduction

Pre-eclampsia is a syndrome that is usually defined as onset of hypertension and proteinuria after 20 weeks of gestation in previously normotensive non-proteinuric pregnant women (Program 2000; Noris et al. 2005). This multisystemic maternal syndrome is one in which the reduced blood perfusion induces a hypoxic state, which affect virtually every major organ system by causing endothelial dysfunction and systemic vasospasm (Roberts and Gammill 2005). Mechanism of increased vascular resistance and hypertension is explained in part by insufficient production of nitric oxide (NO), a potent vasodilator. Some angiogenic inhibitors suppress endothelial nitric oxide synthase (eNOS) expression, which in turn reduces NO production and increases vascular resistance (Baker et al. 1996; Santibanez et al. 2007). Vascular endothelial growth factor (VEGF) is a factor to promote angiogenesis and also cause vasodilation by binding to receptors that active eNOS to produce NO (Wheeler-Jones et al. 1997; Zygmunt et al. 2003; Tammela et al. 2005; Ramesar et al. 2012). Treatment with N(omega)-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), during mid- to late gestation, results in pathological changes similar to those observed in women with pre-eclampsia, such as severe renal vasoconstriction, proteinuria, thrombocytopenia and intrauterine growth retardation (Molar et al. 1994; Khalil and Granger 2002). Pre-eclampsia-like symptoms occurring on early gestational stages affect disease development through the placenta, whereas pre-eclampsia-like symptoms occurring in late pregnancy had a direct impact on the mother and foetus (Ma et al. 2010).

The most commonly used antihypertensive agents cause systemic vasodilatation and can only slightly improve blood pressure control, but have no significant clinical effects on improving renal function and increasing placental blood flow (Ramesar et al. 2012). Sildenafil citrate is a type 5-specific phosphodiesterase inhibitor that potentiates the effects of NO on vascular smooth muscle (Sher and Fisch 2002). Sildenafil citrate may have a role in increasing fetoplacental blood flow in the setting of placental vascular insufficiency and pre-eclampsia (Downing et al. 2004; Maharaj et al. 2009).

This study aimed to investigate the effects of sildenafil citrate on angiogenesis and oxidative stress at the maternal foetal interface on pre-eclampsia-like mouse model induced by L-NAME.

Materials and Methods

Animals

Twenty female mice of the BALB/c strain were selected (weight: 20–24 g, 10–15 weeks old). The animals were housed individually under controlled conditions of temperature (22°C) and lighting (12-h light/dark cycle) and fed with standard mouse chow with water available *ad libitum*. Female mice were mated with males, and the day of plug detection was designated as day 0 of pregnancy. Pregnant mice were transferred to individual cages during the period of pregnancy. Animals were divided into four groups: vehicle control group (VC group, n = 5), sildenafil group (SIL group, n = 5), L-NAME group (LN group, n = 5) and L-NAME+sildenafil group (LN+SIL group, n = 5). All procedures were

approved by the Ethics Committee of Universidad Nacional de Río Cuarto, approval number 45/2011.

L-NAME (Sigma-Aldrich, St. Louis, MO, USA) was prepared in saline solution and stored in a freezer at −20°C. LN and LN-SIL groups received L-NAME subcutaneously (50 mg/kg/day), from day 7 to day 16 of gestation to induce pre-eclampsia-like symptoms.

The sildenafil solution was prepared by grinding Viagra tablets (Pfizer Inc., New York, NY, USA) into powder and dissolving in distilled water (Sarifakioglu et al. 2004). SIL and LN-SIL groups received sildenafil citrate orally (10 mg/kg/day), from day 8 to day 16 of gestation.

Sample collection and morphometric analysis

On day 17, animals were euthanized by cervical dislocation. Thereafter, a laparotomy was performed to expose the uterine horns. The number of developed foetuses and their respective placenta in the right uterine horn were counted, removed, weighed and measured. The following foetal measures were obtained: crown-rump length, bilateral length (right–left) and dorsal–ventral length. The placental measures were recorded: larger diameter, smaller diameter and height. These parameters were obtained by means of vernier caliper.

Placenta and foetuses homogenates

The placenta and foetus of central vesicle in the right uterine horn were homogenized, keeping the tubes in a container with ice flake. The sample was centrifugated at 11 000 × ***g*** at 2°C for 10 min (Heraeus Biofuge Stratos™, Thermo Fisher Scientifc Inc., Waltham, MA, USA). The resulting supernatant was fractionated into Eppendorf tubes and stored at −80°C for subsequent determination of the protein concentration, lipid peroxide formation, activity of the antioxidant enzyme catalase (CAT) and VEGF expression.

Protein concentration of placental homogenates

Protein concentration was determined using a BCA (bicinchoninic acid) protein assay, according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL, USA).

Assessment of oxidative stress

Catalase activity in placental homogenates

CAT activity was measured according to the method of Chance (1954). Briefly, 3 ml of potassium phosphate buffer (pH 7.2) was added to 100 µl of placental homogenates, and then 100 µl of hydrogen peroxide was added to initiate the reaction. The consumption of hydrogen peroxide was monitored by spectrophotometer at 240 nm (Jasco v630bio, Easton, MD, USA) for 2 min with intervals of 10 s. Results were expressed as mmol CAT/mg of protein.

Lipid peroxidation measurements

The amount of lipid peroxidation of the placental homogenates was estimated by measuring the thiobarbituric acid-reactive substances (TBARS) according to the method described by Buege and Aust (1978). The absorbance was measured at 532 nm, and the TBARS values were calculated using a malondialdehyde (MDA) standard curve, prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane. The values were expressed as nmol of MDA/mg of protein.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Placental homogenates were used to determine VEGF expression. Equal amounts of homogenates protein were subjected to SDS-PAGE (Laemmli 1970). A resolving gel with 12% acrylamide was used. After migration, proteins were transferred onto an 0.45-µm Immobilon-P polyvinylidene di-fluoride (PVDF; Millipore, Bedford, MA, USA) membrane. The membrane was blocked in PBS containing 0.1% Tween-20 and 10% non-fat dry milk, washed and incubated with primary antibodies VEGF (A-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at 4°C. After three washes in tris-buffered saline (TBS), the blot was incubated with avidin–biotin–peroxidase complex (Vectastin® ABC, Vector Lab, Burlingame, CA, USA) and revealed with 3, 3′-diaminobenzidine (DAB) (FAST DAB tablet set; Sigma Chemical Co., St Louis, MO, USA). VEGF bands were quantified by densitometry with IMAGEJ software.

Histopathological analysis

The left uterine horn was rapidly removed and stored in 4% formaldehyde in 0.1 M phosphate-buffered solution (PBS) for histopathological analysis. Conventional histological technical was performed on left uterine horn in the central vesicle. The fetoplacental unit samples were fixed with PBS 10% formaldehyde pH 7.4 for 72 h, then dehydrated and embedded in paraffin. Thin sectioning (3–4 µm) was performed using a microtome (Leica Microsystems Inc., Bannockburn, IL, USA). The paraffin sections were rehydrated and then stained with haematoxylin–eosin (H/E) through routine protocols.

Lectin-histochemistry study

Placental sections were stained with BSA-1 lectin (Lectin Kit BK 1000, Vector Laboratories, Inc., Burlingame, CA, USA) to distinguish between foetal capillaries (have an endothelial cell layer) from maternal blood spaces (do not have endothelial cell layer), in the vascularization of the placental labyrinth. Sections were deparaffinized in xylene and rehydrated through an ethanol series to PBS. Endogenous peroxidase activity was blocked using hydrogen peroxide in PBS for

10 min. Two passages were made by microwave for 5 min at 750 W in citrate buffer at pH 6, to increase the exposure of carbohydrate residues. The sections were incubated with biotinylated BSA-1 lectin at room temperature for 2 h. Finally, after three washes were performed with PBS, the samples were treated with the chromogen 3,3′- diaminobenzidine (DakoCytomation, Carpinteria, CA, USA) with hydrogen peroxide. Sections were counterstained with Harris' haematoxylin.

Densitometry analysis of images was performed using IMAGEJ version 1.37 software (National Institutes of Health, Bethesda, MD, USA). The rate between the area occupied by all image and the area marked with BSA-1 lectin was performed to estimate the percentage of foetal endothelium.

Statistical analysis

Statistical analysis was performed with SOFTWARE INFOSTAT®versión 2012e (Di Rienzo et al. 2012). All data were presented as mean ± standard deviation. Data were analysed by two-way ANOVA followed by DGC *post hoc* test. $p < 0.05$ was considered statistically significant.

Results

Morphometric analysis

Foetal parameters

The average number of embryonic vesicles per animal was 7.85 (±1.57). Only two resorptions were observed, and no malformations in the foetuses were identified. The averages of bilateral lengths in the groups treated with L-NAME were significantly decreased compared with the groups not treated with L-NAME ($p < 0.05$). There were no significant differences in weight, crown-rump length and dorsal–ventral length (Table 1).

Placental parameters

The average placental weight in the LN and LN+SIL groups were statistically lower compared with other groups ($p < 0.05$). A statistically significant decrease ($p < 0.05$) in the placental average height was observed in the LN and LN+SIL groups, compared with the SIL and VC groups. There were no significant differences in the statistical analysis of the larger diameter and smaller diameter (Table 2).

Assessment of oxidative stress

There were no statistical differences in catalase activity or lipid peroxidation results between the different groups studied (Table 3).

The concentrations of nmol MDA/mg of protein were incremented in LN group, but there were no significant differences with respect to all other groups. In the VC and SIL groups, the concentrations of mmol CAT/mg of protein were higher than the groups treated with L-NAME, although there were no significant differences between another groups (Table 3).

Western blot analysis

The VEGF expression in the placental tissue of mice at 17 days of gestation was demonstrated by Western blot, although there were no significant differences between the studied groups (Fig. 1).

Endothelial tissue staining

The labyrinth zones were observed under a light microscope. No marked oedema, necrosis or inflammations were observed between the studied groups. A statistically significant decrease in the percentage of foetal endothelium marked with BSA-1 lectin was observed in the LN groups ($p < 0.05$) (Fig. 1).

Table 1. Evaluation of foetal length and weight in different groups

Groups	Weight (mg)	Crown-rump length (mm)	Bilateral length (mm)	Dorsal–ventral length (mm)
Vehicle control	920 ± 54.31	19.36 ± 0.73	7.54 ± 0.63[a]	8.44 ± 0.35
Sildenafil	882 ± 85.85	19.56 ± 1.30	7.90 ± 0.43[a]	8.46 ± 0.37
L-NAME	838 ± 120.08	18.50 ± 1.54	7.10 ± 0.28[b]	8.36 ± 0.52
L-NAME+sildenafil	894 ± 210.78	18.86 ± 2.10	7.08 ± 0.26[b]	8.00 ± 0.56

Data are expressed as mean ± SEM. Two-way ANOVA followed by DGC *post hoc* test. Different letters indicate a significant difference ($p = 0.0163$).

Table 2. Evaluation of placental weight and diameter in different groups

Groups	Weight (mg)	Smaller diameter (mm)	Larger diameter (mm)	Height (mm)
Vehicle control	116 ± 5.48[a]	8.02 ± 0.23	8.48 ± 0.31	2.94 ± 0.09[a]
Sildenafil	116 ± 5.48[a]	7.98 ± 0.40	8.54 ± 0.32	3.12 ± 0.18[a]
L-NAME	100 ± 7.07[b]	7.60 ± 0.30	8.20 ± 0.39	2.30 ± 0.23[b]
L-NAME+sildenafil	92 ± 16.43[b]	7.52 ± 0.27	8.12 ± 0.52	2.32 ± 0.13[b]

Data are expressed as mean ± SEM. Two-way ANOVA followed by DGC *post hoc* test. Different letters indicate a significant difference ($p < 0.05$).

Table 3. Comparison of lipid peroxide formation and activity of the antioxidant enzyme catalase in the placental homogenates

Groups	nmol MDA/mg protein	mmol CAT/mg protein
Vehicle control	211.11 (±17.40)	0.80 (±0.02)
Sildenafil	208.64 (±12.18)	0.80 (±0.04)
L-NAME	231.80 (±6.84)	0.76 (±0.03)
L-NAME+sildenafil	207.69 (±21.92)	0.75 (±0.03)

Data are expressed as mean ± SEM. Two-way ANOVA followed by DGC *post hoc* test.

Discussion

In the present study, we did not see any change in foetal numbers or pathological processes and did not find evidence of malformations in all groups. We showed the absence of oedema, necrosis or inflammation in the histological analysis of the placental labyrinth in the group treatment with sildenafil. Sildenafil was found to have no effect on maternal or foetal morbidity or mortality in this study. Neither the foetal weight nor placental weight or morphometric parameters were altered. The percentage of foetal endothelium labeled with BSA-1 lectin was similar between vehicle control groups and group that received sildenafil. All this evidence suggests that the use of sildenafil did not generate any alteration in the foetal growth. Similar results were obtained by Sasser and Baylis (2010), who demonstrated that treatment with sildenafil in rat with normal gestation did not induce any change in foetal number or development. The treatment with sildenafil in women with intrauterine growth restriction (IUGR) produced no adverse maternal side effects (von Dadelszen et al. 2011).

In recent years, research in animals has demonstrated that NO leads to relaxation of vascular smooth muscles and is a powerful modulator of uterine blood flow (Schäffer et al.2006; Pustovrh et al. 2007; Ramesar et al. 2012). In the underlying vascular smooth muscle, this NO promotes cyclic GMP (cGMP) production and relaxation of the contractile machinery by a number of mechanisms. The duration of action of cGMP in vascular smooth muscle is controlled by the cytosolic enzyme phosphodiesterase-5 (PDE5) (Wareing et al.2006). Dysfunction of the maternal and foetal endothelium with altered activity of eNOS and NO-cGMP pathway has been associated with the development of pre-eclampsia (Salas 1998). It has been suggested that PDE5 inhibitors, such as sildenafil citrate, may improve uterine blood flow via cGMP, mediated endothelial relaxation of uterine vessels (Zoma et al. 2004). Therefore, to observe the effects of sildenafil on the vascularization of the placenta in this study, a model of pre-eclampsia induced by L-NAME was used. In a preliminary report from Ma et al. (2010) observed

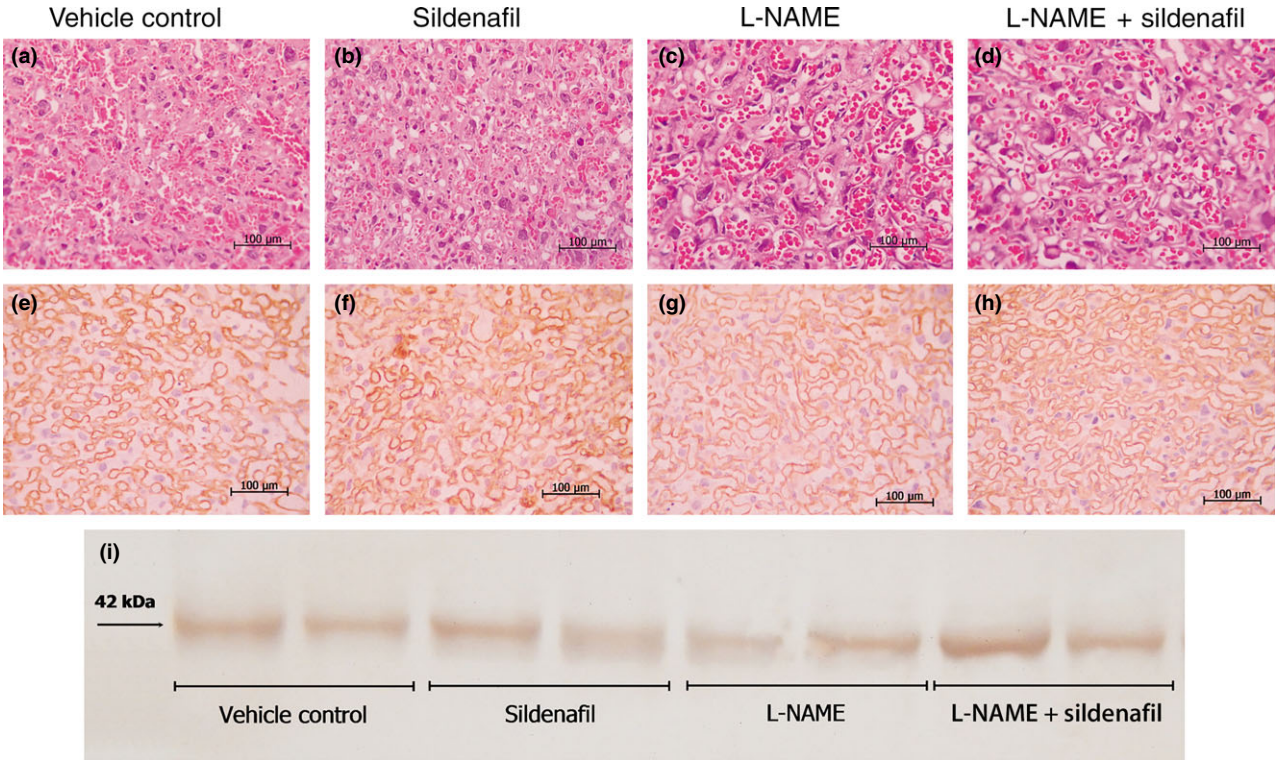

Fig. 1. Photomicrographs of placental labyrinth and Western blot for VEGF on day 17 of gestation. (a–d) the placental labyrinth stained with haematoxylin and eosin (H/E) (400×). (e–h) vascular endothelium stained with BSA-1 lectin and counterstained with haematoxylin (400×). Brown colour areas were used to quantify BSA-1 area by digital image analysis. (i) Western blot for VEGF expression in the placental homogenates

that in a model of pre-eclampsia induced by L-NAME, the pre-eclampsia-like symptoms that occurs at earlier stage (days 6–8) were more likely to be associated with abnormal placental development, whereas late-onset (days 16–20) pre-eclampsia is possibly a maternal disorder. In our study, the evaluation of the parameters related to foetal growth in the pre-eclampsia model, revealed a statistically significant decrease in of bilateral placentas. There were no significant differences in foetal weight and crown-rump length between treated L-NAME groups and the VC group. These observations are agreed with those obtained by Ramesar et al. (2010), who did not find a statistic difference in foetal weight in Sprague Dawley rats treated with L-NAME. Placental parameters showed a decrease in weight and height of the groups treated with L-NAME, and these differences were statistically significant ($p < 0.05$). Ramesar et al. (2010) report similar findings, a decrease in the placental weight in Sprague Dawley rat treatment with L-NAME. In addition, Ma et al. (2010) observed a decrease in placental weight on day 18 of pregnancy, which is induced to pre-eclampsia with L-NAME in the early and middle pregnancy.

The evaluation of the development of blood vessels in the placenta by lectin-histochemistry, showed that the application of L-NAME produced a statistically significant decrease in the percentage of foetal endothelium, but no statistical differences were observed in VEGF expression in homogenates of placental tissue. This decrease in the percentage of foetal endothelium, placental weight and placental height suggests that the application of L-NAME induces a reduction in foetal vascular area and consequently decreases placental blood flow and foetal oxygen supply, which could be causing IUGR. This effect could be mediated by a reduction in NO in foetal trophoblast due to the inhibitory effect of L-NAME on NOS. The decrease in the area of foetal endothelium of the treated animals with L-NAME was reversed when the animals were treated with sildenafil. In these animals, a statistically significant increase in the percentage of foetal endothelium of the placental labyrinth compared to the group receiving only the L-NAME was observed. These findings could be supporting the use of sildenafil to counteract the effect of L-NAME on the placental trophoblast. In this sense, some researchers have shown that sildenafil citrate improves uterine artery blood flow and endometrial development in women undergoing *in vitro* fertilization (Sher and Fisch 2000; Ramesar et al. 2012), as well as having beneficial effects on foetal and vascular parameters in hypertensive pregnant rats (Osol et al. 2005). Also, Dastjerdi et al. (2012) and Wareing et al. (2006) showed that the sildenafil citrate used as a therapeutic agent may improve myometrial perfusion in foetal growth restriction pregnancies by promoting myometrial small artery vasodilatation, decreasing peripheral resistance and increasing flow within the uteroplacental bed. Sildenafil citrate was also showed to enhance vasodilation and improve the endothelial function of myometrial vessels in pregnancies complicated by IUGR (Wareing et al. 2005). Maharaj et al. (2009) reported that sildenafil citrate acts as vasodilator in the fetoplacental circulation via a cGMP-dependent mechanism involving increased responsiveness to NO.

In conclusion, our results indicate that treatment of pre-eclampsia-like mouse model with sildenafil can be used without adverse effects on the concept and its use in the treatment of pre-eclampsia is promising.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Motta C and Grosso C processed the samples and performed all the experimental assays. Picco N and Alustiza F collaborated in the sample processing and the animal management. Molinero D and Bellingeri R collaborated in the sample processing and stress oxidative techniques. Zanuzzi C, Barbeito C and Vivas A advised on the histochemical and lectin-histochemical techniques. This work had completely led by Romanini MC. All authors interpreted the data, critically revised the manuscript and approved the final version.

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