Sildenafil Treatment Ameliorates the Maternal Syndrome of Preeclampsia and Rescues Fetal Growth in the Dahl Salt–Sensitive Rat

Ellen E. Gillis, Jennifer N. Mooney, Michael R. Garrett, Joey P. Granger, Jennifer M. Sasser

Abstract—Preeclampsia, a hypertensive disorder of pregnancy, is detrimental to both mother and fetus. There is currently no effective treatment, but sildenafil, a phosphodiesterase-5 inhibitor, has been proposed as a potential therapy to reduce blood pressure and improve uteroplacental perfusion in preeclamptic patients. We hypothesized that sildenafil would improve the maternal syndrome and fetal outcomes in the Dahl S rat model of superimposed preeclampsia. Dahl S rats were mated, and half received sildenafil (50 mg/kg per day, via food) from day 10 through day 20 of pregnancy. The untreated Dahl S rats had a significant rise in blood pressure and a 2-fold increase in urinary protein excretion from baseline to late pregnancy; however, sildenafil-treated Dahl S rats exhibited ≈40 mm Hg drops in blood pressure with no rise in protein excretion. Sildenafil also increased creatinine clearance and reduced nephrituria and glomerulomegaly. Sildenafil treatment reduced the uterine artery resistance index during late pregnancy in the Dahl S rat and improved fetal outcomes (survival, weight, and litter size). In addition, 19% of all pups were resorbed in untreated rats, with no incidence of resorptions observed in the treated group. Furthermore, tumor necrosis factor-α, endothelin-1, and oxidative stress, which are characteristically increased in women with preeclampsia and in experimental models of the disease, were reduced in treated rats. These data suggest that sildenafil improves the maternal syndrome of preeclampsia and blood flow to the fetoplacental unit, providing preclinical evidence to support the hypothesis that phosphodiesterase type 5 inhibition may be an important therapeutic target for the treatment of preeclampsia.

Key Words: endothelin-1 ▪ oxidative stress ▪ phosphodiesterase-5 inhibitors ▪ pregnancy ▪ tumor necrosis factor-α

Preeclampsia is a leading cause of maternal morbidity and death worldwide, with an estimated 5% incidence in the United States.1,2 Preeclampsia presents after the 20th week of gestation, and it is characterized by hypertension, proteinuria, and endothelial dysfunction. The mechanisms underlying the pathogenesis of preeclampsia are not yet well understood despite the global severity and incidence of this disease, and there are currently no effective treatments other than delivery of the placenta. The consequences of preeclampsia will not end on delivery, as women with preeclampsia, and their offspring, are known to be at an increased risk of cardiovascular disease later in life.3,4 The mechanisms underlying the pathogenesis of preeclampsia are not yet well understood. Therefore, the identification of therapeutic agents that ameliorate the maternal syndrome of preeclampsia, while also promoting the growth and safety of the fetus, are critical.

Recent studies in animal models of pregnancy-induced hypertension have suggested a potential therapeutic role of sildenafil citrate, a phosphodiesterase type 5 (PDE5) inhibitor, to improve maternal and fetal outcomes in preeclampsia.5–7 Normally, PDE5 catalyzes the breakdown of cGMP, and the inhibition of PDE5 causes a sustained response from cGMP, improving endothelial function and vasodilation. Previous studies have shown that sildenafil has no adverse effects on maternal or fetal parameters during pregnancy in preeclamptic human patients8 or in healthy Sprague Dawley rats.9 In the Reduced Uterine Perfusion Pressure model of pregnancy-induced hypertension, sildenafil treatment significantly attenuated the hypertension during pregnancy, but had no effect on pup size or angiogenic factors.5 Sildenafil treatment was found to improve fetal growth and normalize umbilical artery Doppler waveforms in the preeclamptic catechol-O-methyl transferase knockout−/− mouse model.5 However, sildenafil administration in the L-NAME model of intrauterine growth restriction and preeclampsia has yielded mixed results with one study reporting that sildenafil treatment resulted in a decrease in pup weight10 and another study demonstrated that sildenafil improved both maternal and
fetal outcomes. Sildenafil treatment was not able to prolong pregnancy in preeclamptic women in a small clinical trial; however, this may have been because of late administration and suboptimal dosing. Thus, further studies are necessary to determine the potential efficacy and mechanisms of sildenafil in pregnancies complicated by hypertension and fetal growth restriction.

Previous studies in our laboratory have shown that the Dahl salt-sensitive (Dahl S) rat spontaneously exhibits a preeclamptic phenotype during pregnancy, characterized by increased blood pressure, severe exacerbation of proteinuria, increased fetal demise, and decreased pup size. In this study, we tested the hypothesis that sildenafil treatment beginning on gestational day (GD) 10 (midpregnancy in the rat) would improve fetal outcomes and ameliorate the maternal syndrome in this model of superimposed preclampsia.

Methods

Animals

Dahl salt–sensitive S (SS/jr) rats were obtained from the colony maintained by Dr Garrett at the University of Mississippi Medical Center, and they were fed normal chow (TD7034, 0.3% NaCl, Harlan Teklad, Madison, WI). Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were monitored by the University of Mississippi Medical Center Institutional Animal Care and Use Committee. Sildenafil citrate (BIOTANG, Inc) was administered at 50 mg/kg per day to a subset of rats in a gel diet beginning on GD 10 through GD 20. Blood pressure was measured via telemetry, and uterine artery resistance was measured via Doppler ultrasound. On GD 20, rats were anesthetized for tissue collection, and renal histology and biochemical assays were performed as previously described.

All data are presented as means±SE. Statistical analyses were performed by either Student’s t test (between the treated and the control groups) or repeated measures ANOVA (mean arterial pressure [MAP] and proteinuria data) using Sigma Plot 12 (Systat Software, Inc). Means were considered significantly different if P<0.05.

Results

Sildenafil Treatment Increases Urinary cGMP Excretion

In healthy pregnancy in women and in rats, cGMP excretion is increased because of increased nitric oxide (NO) production; however, this normal response to pregnancy is absent in the Dahl S rat. We found that urinary cGMP excretion is decreased in late pregnancy compared with virgin control Dahl S rats (52.7±5.1 versus 77.3±8.8 nmol/d, P<0.05; E.E. Gillis and J.M. Sasser, unpublished data, 2015). To confirm the efficacy of treatment with sildenafil at the dose of 50 mg/kg per day, we examined urinary excretion rates of cGMP in treated rats. Sildenafil treatment significantly increased cGMP excretion (77.0±8.6 nmol/d, P<0.05 versus control pregnant, Figure S1 in the online-only Data Supplement).

Increased MAP in the Pregnant Dahl S Is Reversed With Sildenafil Treatment

MAP was similar at baseline in the control and treated Dahl S rats (159.5±10.1 mmHg versus 162.5±12.8 mmHg). During the course of pregnancy, MAP steadily increased in the control group, with a significant increase during late pregnancy. Sildenafil treatment resulted in an immediate decrease in MAP beginning on GD 10, which was sustained throughout late pregnancy (Figure 1A). In Figure 1B, the change in MAP from baseline to late pregnancy reveals the efficacy of sildenafil at reducing blood pressure during pregnancy in the Dahl S rat, with ≈10 mmHg rise in the control group and ≈38 mmHg drop in the treated rats.

Improved Renal Function After Sildenafil Treatment

Urinary protein excretion was similar at baseline between the control and the treated groups (103.5±18.1 versus 96.0±19.3 mg/d). Normally during the course of pregnancy, the Dahl S rat exhibits an increase in proteinuria, as shown in Figure 2A. However, urinary protein excretion remained unchanged throughout the course of pregnancy in the treated Dahl S rats, resulting in a significant difference between groups during mid and late pregnancy. In addition to preventing the rise in proteinuria during pregnancy in the Dahl S dams, sildenafil treatment also resulted in substantial increases in renal function in the pregnant Dahl S rats. Creatinine clearance was significantly increased, with a corresponding decrease in plasma creatinine, in the treated rats (Figures 2B and 2C). Urinary nephrin excretion was also decreased after sildenafil treatment (Figure 2D).

Figure 1. Blood pressure decreases with sildenafil treatment in the pregnant Dahl S rat. A, Mean arterial pressure (MAP) in control (CON) and treated (SILD) groups at baseline (BL) and throughout pregnancy. B, The change in MAP from baseline to late pregnancy in control and sildenafil–treated rats; n=6 to 8, *P<0.05 vs control, †P<0.05 vs baseline.
During pregnancy, the Dahl S rat exhibits glomerulomegaly, but sildenafil treatment prevented this renal damage. Glomerular area and diameter did not increase during pregnancy in the treated group compared with the untreated controls (Figure 3).

Sildenafil Attenuates the Elevated TNF-α, Endothelin-1, and Oxidative Stress in the Pregnant Dahl S Rat

Tumor necrosis factor-α, endothelin-1, and oxidative stress are characteristically increased in preeclamptic women and in animal...
models of preeclampsia, including the Dahl S rat. We previously published that tumor necrosis factor-α levels are increased in the Dahl S pregnancy. We have also observed increases in urinary excretion of endothelin-1 (4.28±0.75 versus 7.61±1.64 pg/d, P=0.05; E.E. Gillis and J.M. Sasser, unpublished data, 2015) and isoprostanes (8.42±2.18 versus 30.61±3.69 ng/d, P<0.05; E.E. Gillis and J.M. Sasser, unpublished data, 2015) during pregnancy in the Dahl S rats compared with the virgin age-matched controls. Sildenafil treatment resulted in a significant reduction of tumor necrosis factor-α in both the plasma (55% reduction, Figure 4A) and the placenta (26% reduction, Figure 4B). Sildenafil-treated rats also had significant reductions in urinary excretion of endothelin-1 (49% reduction, Figure 4C), TBARS (thiobarbituric acid reactive substances; 30% reduction, Figure 4D), and isoprostanes (82% reduction, Figure 4E).

**Uterine Artery Resistance Index Is Normalized With Sildenafil Treatment**

During normal pregnancy, uterine artery resistance index (UARI) is elevated during midpregnancy (ranging from 0.6 to 0.8), but UARI is expected to decrease during late pregnancy in response to systemic vasodilation. However, in the Dahl S rat UARI remains elevated (0.71±0.09) during late pregnancy, as shown in Figure 5A. UARI was normalized with the administration of sildenafil in the Dahl S rat, with a significant reduction in UARI during late pregnancy (0.52±0.04). Sildenafil treatment also markedly affected the uterine artery waveform characteristics during late pregnancy. The Dahl S normally exhibits characteristic notching in the waveforms, as shown in Figure 5B. This notching is absent in the sildenafil-treated Dahl S rats (Figure 5C).

**Sildenafil Improves Dahl S Fetal Growth**

The adverse uterine environment causes intrauterine growth restriction during preeclampsia, resulting in decreased fetus size in human preeclamptic patients and preeclamptic animal models, including the Dahl S rat. Sildenafil treatment significantly improved fetal growth in the Dahl S rat. Treated rats had significantly larger pups, as measured by an increase pup weight (Figure 6A) and an increase in pup length (Figure 6B). Although litter size did not significantly differ between the 2 groups (Figure 6C, P=0.198), there was a dramatic decrease

---

**Figure 4.** Sildenafil attenuates the elevated tumor necrosis factor (TNF)-α in the Dahl S plasma and placenta. TNF-α is decreased in both the (A) plasma and (B) placenta in the treated group (SILD) compared with the control group (CON). Urinary excretion rates of endothelin (ET)-1 (C), thiobarbituric acid reactive substances (TBARS) (D), and isoprostanes (E) are also significantly attenuated in the SILD compared with CON; n=6 to 9, *P<0.05 vs control.
Discussion

Preeclampsia is a serious disorder of pregnancy, and there is a critical need for new therapeutics to simultaneously treat the maternal syndrome and foster fetal growth. The goal of this study was to determine if PDE5 inhibition is a potential therapeutic target in preeclampsia, and we report novel findings that sildenafil treatment is able to ameliorate the maternal syndrome and rescue fetal growth in the preeclamptic Dahl S rat. The major findings of this study were that sildenafil treatment during preeclamptic pregnancy resulted in (1) sustained reductions in MAP with improved renal function, (2) decreased UARI, and (3) significantly increased fetal growth.

Hypertension and proteinuria are 2 of the hallmark features of preeclampsia, and have been previously described by our laboratory in the preeclamptic Dahl S rat. Sildenafil treatment resulted in immediate and sustained decreases in blood pressure and also improved renal function in the Dahl S rat. Our findings suggest that the reduced blood pressure and renal function in rats treated with sildenafil may be because of decreased levels of tumor necrosis factor-α, endothelin-1, and oxidative stress, 3 factors that have been implicated in the pathogenesis of preeclampsia in several studies. Furthermore, these results are in line with previous studies in other animal models of preeclampsia. Sildenafil also improves the maternal syndrome of preeclampsia observed in the Reduced Uterine Perfusion Pressure model of pregnancy-induced hypertension and significantly decreases blood pressure, proteinuria, and sFlt-1 (soluble fms-like tyrosine kinase-1) and sEndoglin levels in the L-NAME rat model of preeclampsia. Furthermore, studies in human patients have reported similar effects on the maternal syndrome of preeclampsia. Several case studies have reported that patients with idiopathic pulmonary arterial hypertension have improved maternal and fetal outcomes when given sildenafil during pregnancy. In an early clinical trial, sildenafil treatment was not effective in prolonging pregnancy in preeclamptic women, despite decreasing maternal blood pressure. This negative outcome on time to delivery may be because of insufficient plasma concentrations of sildenafil because of the slow dosing regimen that was needed to ensure patient safety, the lack of early intervention and, potentially, differences in the pharmacokinetic profile of sildenafil in preeclamptic women. Therefore, additional studies are needed to determine the efficacy of sildenafil in patients with preeclampsia, and in particular those with preeclampsia superimposed on pre-existing hypertension. Currently in the planning stages, the Dutch STRIDER (Sildenafil Therapy in Dismal Prognosis Early-Onset Intrauterine Growth Restriction) trial proposes to further investigate the potential therapeutic role of sildenafil in pregnancies complicated by intrauterine growth restriction.

Sildenafil is a PDE5 inhibitor that prolongs the effects of NO-cGMP signaling by inhibiting the breakdown of the second messenger, cGMP. Potentiation of NO-cGMP signaling in the vasculature causes vasodilation, resulting in increased blood flow. Decreased NO activity is a characteristic feature in animal models of preeclampsia, and preliminary studies in sheep have reported that PDE5 is localized in the maternal portion of the uteroplacental unit. Furthermore, inhibition of PDE5 by sildenafil significantly enhanced vasorelaxation in myometrial small arteries taken from women with preeclampsia and fetal growth restriction. Vasorelaxation of the uterine arteries could enhance uteroplacental blood flow to improve fetal outcomes. This is consistent with the results reported in this study, in which we report that sildenafil treatment decreases UARI and prevents notching in the uterine artery Doppler waveforms, suggesting an improvement in uteroplacental perfusion during late pregnancy. The effects on UARI reported here have previously been described in human preeclamptic patients as well. In 1 case study, sildenafil treatment significantly improved uterine artery pulsatility index in a patient having fetal growth restriction.

The beneficial effects of sildenafil treatment in the Dahl S dams are also advantageous for the Dahl S pups. Sildenafil-treated Dahl S dams had significantly larger pups with a dramatic decrease in the rate of fetal demise, potentially as
Hypertension
March 2016

a result of improved uteroplacental blood flow after the dilation of the uterine arteries. A similar effect on fetal growth by sildenafil treatment has previously been described in the catechol-O-methyl transferase knockout−/− mouse model, corresponding with a decrease in umbilical artery resistance index.5 Isolated case studies in women presenting with intrauterine growth restriction have previously reported substantial increases in fetal weight after sildenafil administration.27,28 Sildenafil’s beneficial effects on the fetus are vital because preeclampsia is a disease that is simultaneously affecting 2 patients, the mother and the fetus. It has therefore been proposed that restoration of the vasodilatory NO pathway both systemically and specifically in the uteroplacental vasculature via PDE5 inhibitors, such as sildenafil may be a novel, effective therapeutic option for this disease.

Perspectives
In this study, sildenafil treatment resulted in improved fetal growth and significant amelioration of the maternal syndrome in a spontaneously preeclamptic rat model, the Dahl S rat. These data suggest that sildenafil improves blood flow to the fetoplacental unit despite lowering maternal blood pressure, providing preclinical evidence to support the hypothesis that phosphodiesterase-5 inhibition is a therapeutic target for the treatment of preeclampsia. Future work is needed to determine the effects of sildenafil treatment on placental development and angiogenesis to further elucidate the mechanisms by which PDE5 inhibition and enhancement of NO-cGMP signaling are beneficial to both the mother and the fetus during preeclamptic pregnancy.

Acknowledgments
We thank Ashley Johnson for expert technical assistance.

Sources of Funding
Research reported in this publication was supported by the National Institute of Diabetes and Digestive and Kidney Diseases under award number K01 DK095018 (J.M. Sasser), NIGMS under Award Number P20GM104357 (J.M. Sasser), and National Heart, Lung, and Blood Institute under award numbers T32HL105324 (E.E. Gillis) and R01HL094446 (M.R. Garrett). The content is solely the responsibility of us and does not necessarily represent the official views of the National
Novelty and Significance

What Is New?
- This is the first study to show the beneficial cardiovascular and renal protective effects of phosphodiesterase 5 inhibition in a spontaneous animal model of preeclampsia.

What Is Relevant?
- These results provide preclinical evidence the phosphodiesterase type 5 inhibition may be a suitable therapeutic target in the treatment of preeclampsia.

Summary
Preeclampsia is a leading cause of maternal morbidity and death worldwide, and there is currently no effective treatment available. Here, we show that sildenafil, a phosphodiesterase 5 inhibitor, significantly improved maternal cardiorenal health and rescued fetal growth in a spontaneous animal model of preeclampsia.

Disclosures
None.

References

Institutes of Health. This work was also supported by American Heart Association PRE22660009 (E.E. Gilles), the University of Mississippi Medical Center Intramural Research Support Program (J.M. Sasser), the Robert M. Hearin Foundation (M.R. Garrett), and the Dean Franklin Young Investigator Award from Data Sciences International/American Physiological Society (J.M. Sasser).
Sildenafil Treatment Ameliorates the Maternal Syndrome of Preeclampsia and Rescues Fetal Growth in the Dahl Salt–Sensitive Rat
Ellen E. Gillis, Jennifer N. Mooney, Michael R. Garrett, Joey P. Granger and Jennifer M. Sasser

Hypertension. published online January 4, 2016;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2016/01/04/HYPERTENSIONAHA.115.06071

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2016/01/04/HYPERTENSIONAHA.115.06071.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
Sildenafil treatment ameliorates the maternal syndrome of preeclampsia and rescues fetal growth in the Dahl Salt Sensitive rat

Ellen E. Gillis¹, Jennifer N. Mooney¹, Michael R. Garrett¹,², Joey P. Granger³, Jennifer M. Sasser¹

¹Department of Pharmacology and Toxicology, ²Department of Medicine, ³Department of Physiology and Biophysics,

University of Mississippi Medical Center, Jackson, Mississippi
Expanded Methods

Animals
Dahl salt-sensitive S (SS/jr) rats were obtained from the colony maintained by Dr. Michael Garrett at the University of Mississippi Medical Center. All rats were fed normal chow (TD7034, 0.3% NaCl, Harlan Teklad, Madison, WI) and water ad libitum on a 12-hour light/dark cycle. All rats were time-bred, with the presence of sperm in vaginal smears indicative of gestational day (GD) 1. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were monitored by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

Sildenafil Treatment
Sildenafil citrate (BIOTANG, Inc.) was administered at 50 mg/kg/day to a subset of rats in a gel diet beginning on GD 10 through GD 20. The dose has previously been shown to cause a significant increase in plasma cGMP with no effects on fetal growth in pregnant Sprague Dawley rats \(^1\). The gel diet contained all of the required nutrients and was made by dissolving 242 g of powdered Custom AIN 76C low-nitrate diet (0.3% NaCl, MP Biomedicals, Solon, OH) and 6 g of agar in 275 ml of water. We previously observed that the addition of sildenafil did not affect food intake. In this study, rats were weighed regularly, and the gel diet was prepared every other day to account for changes in body weight and food consumption over the course of pregnancy.

Mean Arterial Blood Pressure Measurements
Rats were implanted with telemetry devices (Data Sciences, Inc.) via the femoral artery for continual blood pressure monitoring at ~16 weeks of age as previously described \(^1\). After a 10-day recovery period, baseline measurements were obtained prior to mating. Blood pressure measurements were recorded during early, mid, and late pregnancy (GD 4, GD 14, and GD18, respectively), and immediately preceding the start of sildenafil treatment (GD9).

Uterine Artery Resistance Index
Pregnant dams underwent Power Doppler velocimetry measurements on GD18 at an imaging station with a Vevo 770 unit (FUJIFILM VisualSonics, Inc.) using a 30-Hz transducer at an insonating angle <30 degrees. The uterine artery Doppler waveform was recorded, and the peak systolic flow velocity (PSV) and end-diastolic flow velocity (EDV) values were measured to calculate the uterine artery resistance index (UARI) using the formula UARI= (PSV-EDV)/PSV. UARI was determined for the uterine artery bilaterally at 3 different locations along the uterine artery in each rat, and the mean UARI was recorded.

Tissue Collection
Rats were euthanized on GD20 while on isoflurane anesthesia (Piramal Healthcare). A terminal blood sample was obtained from the abdominal aorta, and the organs were subsequently perfused blood-free with saline. The number
of viable and resorbed fetuses was recorded for each uterine horn. For each viable fetus, placental weight, fetus weight, and fetus length were recorded.

Renal Histology

A section of the left kidney was fixed in 10% buffered formalin and embedded in paraffin, cut into 4 μm sections, and stained with Masson’s trichrome.

Glomerular area and diameter were measured in 20 randomly selected glomeruli from each slide (n=10 dams per group, x40 magnification, Nikon Eclipse 80i microscope with digital camera, Nikon Instruments) using Nis-Elements image analysis software (version 3.03; Nikon Instruments).

Plasma and Urinary Measurements

Rats were placed in metabolic cages for 24-hour urine collection prior to mating for baseline measurements, and during early, mid, and late pregnancy (GD 6, GD 13, and GD19, respectively). Urinary protein excretion was determined by Bradford Assay (Bio-Rad Laboratories). Proteinuria was defined as >20 mg protein/24 hours. Nephrin (Exocell), endothelin-1 (R and D Systems), TBARS and 8-isoprostane (Oxford Biomedical), and cGMP (Cayman Chemical) excretion rates were determined by commercially available assays according to manufacturer instructions. Plasma and urine creatinine concentrations were measured by picric acid method as previously described ².

TNF-α ELISA and Placental Homogenization

TNF-α was measured in plasma and placental homogenates via commercially available ELISA (R&D Systems) according to manufacturer’s instructions. One placenta per pregnant rat was homogenized for analysis. Total soluble protein was extracted in radioimmunoprecipitation assay (RIPA) lysis buffer containing PMSF in dimethyl sulfoxide, sodium orthovanadate, and a protease inhibitor mixture (Santa Cruz Biotechnology, Inc.). DC protein assay (Bio-Rad Laboratories) was used to determine total protein concentration for each homogenized placenta.

Statistical Analysis

All data are presented as mean ± SE. Statistical analyses were performed by either Student’s t-test (between the treated and control groups) or repeated measures ANOVA (MAP and proteinuria data) using Sigma Plot 12 (Systat Software, Inc.). Means were considered significantly different if p<0.05.

References

Figure S1: Sildenafil treatment increases urinary excretion of cGMP in the pregnant Dahl S rat. n=4-5, *p<0.05 vs control.