

Beneficial Effects of Metolazone in a Rat Model of Preeclampsia

Gabriella Pridjian, Candice Pridjian, Svitlana Danchuk, Monica Ianosi-Irimie, Hop V. Vu,
Jules B. Puschett

Departments of Obstetrics/Gynecology (GP) and Medicine, Section of Nephrology (CP,
SD, MI, HVV, JBP), Tulane University School of Medicine, New Orleans, Louisiana

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Correspondence to: Gabriella Pridjian, M.D., Department Of Obstetrics & Gynecology,
SL-11, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA
70112-2699

Phone: (504) 988-5217;

Fax:

E-mail: Gabriella.Pridjian@Tulane.edu

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ABBREVIATIONS: ECF, extracellular fluid; DOCA, desoxycorticosterone acetate;
IUGR, intrauterine growth restriction; BP, blood pressure; NO, nitric oxide; NOS, nitric
oxide synthase; NO_x, nitrite/nitrate; MBG, marinobufagenin

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Abstract

Preeclampsia is a disorder, which continues to exact a significant toll with respect to maternal morbidity and mortality as well as fetal wastage. Furthermore, the treatment of this disorder has not changed significantly in 50 years and is unsatisfactory. The use of diuretics in this syndrome is controversial because there is a concern related to potential baleful effects of volume contraction leading to a possible further decrement in the perfusion of the maternal-fetal unit. Metolazone is a diuretic/antihypertensive agent, which has a therapeutic effect on blood pressure (BP) in human essential hypertension without causing a natriuresis. We administered the drug in non-diuretic doses in a rat model of preeclampsia previously developed in this laboratory. The drug reduced BP without an accompanying natriuresis. Although there was a trend toward an improvement in intrauterine growth restriction, as determined by litter size and the number of pups demonstrating malformations, the values did not reach statistical significance. We conclude that metolazone, in low dosage, is an effective antihypertensive in this rat model. These studies have implications for the treatment of the human disorder.

Introduction

Preeclampsia affects from 3 to 10% of all pregnant women in the United States and worldwide (Lenfant and Zuspan, 1990; Pridjian and Puschett, 2002a). Yet it is still a disease treated by symptom-directed therapy, usually magnesium sulfate and hydralazine or labetalol. Specific therapy directed to the underlying cause has not been developed.

The emerging concept regarding the etiology of preeclampsia is that it is likely multifactorial (Page, 1972; Ness and Roberts, 1996; Pridjian, 1999; Pridjian and Puschett, 2002b). Currently, there is no one satisfactory treatment regimen, which is directed at the underlying cause (Pridjian and Puschett, 2002b), although certain treatments have been proposed in certain preeclamptic subtypes (Saisto et al., 2004). We believe that successful definitive treatment of preeclampsia will be developed aimed at different subtypes.

Women with preeclampsia are volume expanded, as are all pregnant women (Rovinsky and Jaffin, 1965; Scott, 1972). However, the added fluid resides in the interstitial, rather than intravascular, extracellular compartment (Gallery, 1999). In the past, diuretics were avoided in preeclampsia so as not to cause further volume contraction of the intravascular compartment and potential compromise of the maternal-fetal circulation. However, volume contraction and decreased perfusion of the placenta with diuretics has never been proven.

We have postulated that at least some forms of preeclampsia are related to excessive expansion of the extracellular fluid (ECF) volume (Ianosi-Irimie et al., 2005; Vu et al., 2005). We hypothesize that women who develop this type of preeclampsia

have an acquired or congenital defect in sodium transport, which prevents them from excreting the excess sodium. In most women, this defect would not become manifest until the patient is challenged with the 40-50% expansion of ECF volume that accompanies normal pregnancy (Gallery, 1999). The expansion process may actually result in the elaboration of one or more circulating factors (VanWijk et al., 2000; Hayman et al., 2001; Vu et al., 2005), which have both natriuretic and vasoconstrictive properties (Graves and Williams, 1984; Morris et al., 1988; Hilton et al., 1996; Lopatin et al., 1999). The authors have developed an animal model of preeclampsia (Ianos-Irimie et al., 2005), which has many of the phenotypic characteristics of the human disease (Outland et al., 2005). It consists of two manipulations: 1) replacement of the drinking water of the pregnant rat with saline, and 2) administration of the mineralocorticoid, desoxycorticosterone acetate (DOCA) to ensure that the excess sodium is retained. Under these circumstances, the animals develop hypertension, proteinuria and intrauterine growth restriction (IUGR) (Vu et al., 2005).

Metolazone is a diuretic/antihypertensive agent that has been on the market for a number of years. It is a quinethazone derivative, a congener of the thiazide drugs (Puschett, 1972). It is considered safe in human pregnancy; specifically rated Class B by the FDA use in pregnancy rating scale (Physician's Desk Reference, 2000). In low doses, the drug has antihypertensive activity with minimal diuresis and natriuresis (Puschett J *Unpublished observations*). We reasoned that, if this were the case, it might be effective in treating our "preeclamptic" rats without compromising ECF, particularly intravascular volume.

Methods

Experimental Protocols. Female Sprague-Dawley rats (200-250 g) (Harlan, Indianapolis, Indiana) were housed in metabolic cages for a non-pregnant, 24-hour, baseline urine collection. A control (non-pregnant) group was established for comparison (n=8). The remaining animals were then mated with male rats (275-300 g) and pregnancy was confirmed by the presence of vaginal plugs. Pregnant females were isolated from the males and randomly divided into the following groups: normal pregnant (NP) (n=10) and pregnant + DOCA + saline (PDS) animals (n=10). PDS rats were injected intraperitoneally (IP) with 12.5 mg of a depot form of DOCA at the time of mating, followed by a 6.5 mg injection on a weekly basis. In this group, drinking water was replaced with 0.9% saline. In addition, a separate group of PDS animals were given daily metolazone diluted in saline by gavage at 50 µg/kg body weight (PDSM) (n=15). The dose of metolazone was chosen because it corresponds to a low normal dose for humans. All animals were allowed free access to standard rat chow (Lab Diet 5001 Laboratory Rodent Diet), maintained on a 12:12 hour light: dark cycle and acclimatized for one week prior to being studied. Animal care was conducted in accordance with institutional guidelines.

Blood Pressure, Sodium Excretion Measurements. Systolic blood pressure (BP) was measured utilizing a tail-cuff method as previously described (Ianosi-Irimie et al., 2005) and 24-hour urine collections were measured/collected daily. Sodium excretion was measured by flame photometry (Instrumentation Laboratory #943).

Once animals in the PDS and PDSM groups became hypertensive (at approximately day 6-8), metolazone treatment commenced. The metolazone dose was adjusted daily

according to the BP and to the last 24-hour sodium (Na) excretion measurement to insure that metolazone was administered in non-natriuretic doses. For example, if the Na concentration in the 24-hour urine obtained just prior to dosing was either greater than the Na concentration obtained the day before, or greater than the sodium concentration of the untreated PDS group, the dose of metolazone administered would be decreased by 5-8%. If the BP was elevated but there was no evidence of natriuresis, the metolazone dose would be increased by 5-8%. The dose was thus individualized but ranged from 35-80 $\mu\text{g}/\text{kg}$ body weight during the course of the experiment.

Protein, Creatinine, Nitrite/nitrate Assays. At day 19-20, a 24-hour urine was collected for protein, creatinine, and nitric oxide (NO) determinations. On the 20th day of pregnancy animals were humanely euthanized and blood samples were taken. Pups and placentas were separated and any pup malformations were noted.

Urinary protein was measured using the pyrogallol red method with a Total Protein Kit (Sigma-Aldrich). Blood and urine creatinine levels were measured using the picric acid method with a Beckman Creatinine Analyzer (Beckman Coulter). Nitrite/nitrate (NO_x) measurements in sera and in 24-hour urines were performed utilizing sulfanilamide and N-(*n*-naphthyl)-ethylenediamine with a NO Colorimetric Assay (Roche Diagnostics). Hematocrit was measured using an Autocrit Ultra 3 centrifuge.

Immunoblotting Analyses. Kidneys were excised, weighed, dissected and washed in ice-cold saline buffered with 10 mM Tris-HEPES before removal of the cortex and medulla. Kidney slices (cortex and medulla) were homogenized in 50 mM mannitol buffered with 20 mM Tris-HEPES in washed sea sand (Fisher). Cell extracts were obtained after centrifugation (1000 g, 10 min) and protein was measured with a BCA

assay kit (Pierce) with bovine albumin as the standard. Protein samples for immunoblotting analysis (10-15 μ g protein/sample) were prepared in Novex LDS sample buffer (Invitrogen, Carlsbad, CA), separated on 7% Tris-Acetate NuPAGE gels (Invitrogen) (Laemmli, 1970) and transferred to a 0.2 μ m nitrocellulose membrane (BioRad). The membranes were blocked (PBS, 0.5% Tween-20, 5% milk) for 1h at room temperature, briefly rinsed (PBS, 0.5% Tween-20) and stained with monoclonal anti-nitric oxide synthase, endothelial (eNOS) or neuronal (nNOS), antibody (BD Transduction Laboratories). Membranes were then washed and incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham) for 1 h. The chemiluminescent detection was performed by using ECL Western Blotting detection reagents (Amersham Biosciences) and autoradiographs were digitized by QuantiScan (Biosoft Inc., Ferguson, MO). The results were normalized for β -actin (monoclonal anti- β -actin antibody, clone AC-15, Sigma).

Statistical Analysis. Values are presented as mean \pm S.E.M. Statistical comparison analyses were performed using covariant analysis for multiple determinations. *P* value of less than 0.05 was considered significant.

Results

Mean tail-cuff BP in the control, non-pregnant group (C) varied between 97 and 109 mm Hg (Fig. 1). None of the mean values for this group significantly differed from each other at any time during pregnancy. The normal pregnant (NP) mean BP fell from an initial mean value of 109 ± 6 to 88 ± 1 mm Hg after 19 days of pregnancy ($p < 0.01$). This decline is reminiscent of the fall in BP seen in human pregnancy as gestation proceeds. The PDS group (that is, animals rendered “preeclamptic”) displayed a BP increase from 103 ± 6 to 126 ± 1 mm Hg ($p < 0.001$). In the metolazone-treated preeclamptic animals (PDSM group) a statistically significant decrease in BP was noted (pretreatment mean BP 110 ± 4 mm Hg, post treatment BP 94 ± 4 mm Hg, $p < 0.05$). The latter value was similar to that noted in the normal pregnant group ($p > 0.05$). The decrement in tail-cuff BP of the PDSM animals was observed by 24-hours after initiation of metolazone. There were no statistically significant differences in BP between any of the four groups of animals at time t_0 . However, at 4-7 days of gestation (t_1) mean BP values in PDS and PDSM groups were significantly increased (113 ± 4 and 110 ± 4 mm Hg, respectively), and these changes are in contrast to a decrease in the NP group (94 ± 4 mm Hg) (NP vs. PDS $p < 0.01$; NP vs. PDSM $p < 0.05$). At 10-13 days of gestation (t_2) BP of the PDSM animals was decreased (94 ± 5 mm Hg) after metolazone administration, but PDS BP continued to increase (118 ± 2 mm Hg) (PDS vs. PDSM $p < 0.001$).

Animal weight did not demonstrate any significant difference among the pregnant groups of animals (NP, PDS, PDSM) throughout the course of the experiments (Fig. 2).

Twenty-four hour sodium (Na) excretion values (mmol Na / 24 h) in the control and normal pregnant animals were similar throughout the experiment (C: 3.0 ± 0.1 , NP: 4.2 ± 0.3 , day 19, $p > 0.05$) (Fig. 3). The animals in the PDS and PDSM groups had significantly higher, but similar 24-hour urinary Na excretion values (15.9 ± 2.2 and 16.1 ± 3.1 , respectively, on day 19, $p > 0.05$). The day 19 urinary Na mean values were consistent with sodium excretion rates obtained throughout the experiment once the animals were rendered hypertensive.

The normal pregnant animals excreted more protein (mg / 24 h) than non-pregnant controls (NP: 4.9 ± 0.5 vs. C: 2.5 ± 0.4 ; $p < 0.05$) (Fig. 4). The PDS animals demonstrated a significantly greater urinary protein excretion (8.2 ± 1.0) when compared to NP and C ($p < 0.01$ and $p < 0.001$, respectively). The PDSM group did not show a decrement in protein excretion (8.0 ± 1.4) compared to the PDS animals.

Serum creatinine values (mg/dL) in NP (0.6 ± 0.03), PDS (0.5 ± 0.1), and PDSM (0.6 ± 0.1) groups were not different from each other, but were significantly lower than the non-pregnant C group (0.8 ± 0.1 , $p < 0.05$ vs. NP and PDSM, and $p < 0.01$ vs. PDS). Creatinine clearance (ml/min) was found to be increased (vs. non-pregnant control animals) in the NP and PDS groups ($p < 0.05$ in each case), but just missed statistical significance although demonstrating a numerical increase in the PDSM rats (C: 0.8 ± 0.1 , NP: 1.6 ± 0.2 , PDS: 1.6 ± 0.4 , PDSM: 1.4 ± 0.1).

Blood NO as estimated by NO_x measurements ($\mu\text{mol/l}$) was significantly higher in the NP (42.4 ± 2.1 , $p < 0.001$), PDS (34.3 ± 2.9 , $p < 0.01$), and PDSM (31.7 ± 1.3 , $p < 0.05$) groups when compared to the non-pregnant C group (25.6 ± 2.4) (Fig. 5A). PDS animals had a significantly lower blood NO_x when compared to NP ($p < 0.01$). PDSM

animals had a similar lowering when compared to NP ($p < 0.001$). There was no difference noted between the PDS and PDSM groups ($p > 0.05$).

There was no difference in urinary NO excretion (expressed in nM NO_x/mg creatinine) between the C and NP groups (71.9 ± 11.8 vs. 48.3 ± 10.5), but there was an increase in both PDS (151.1 ± 25.9) and PDSM (102.7 ± 19.1) groups vs. NP ($p < 0.001$, $p < 0.05$, respectively) (Fig. 5B). An increase in the PDS animals' urinary NO_x vs. controls was also present ($p < 0.01$). There was no difference in levels between PDS and PDSM groups ($p > 0.05$).

A determination of eNOS and nNOS in Western blots from kidney cortex and medulla demonstrated no differences between the NP, PDS and PDSM groups (data not shown).

Pup number in the PDS group was significantly decreased when compared to the NP rats (13.9 ± 0.4 vs. 11.2 ± 0.9 , $p < 0.05$). However, despite a trend towards an improvement in the PDSM rats (13.4 ± 0.9), this value did not reach statistical significance ($p > 0.05$). With regard to malformations, none were noted in the NP group. The number of developmental malformations in the PDS animals was increased compared to NP (Fig. 6A, B). These consisted of grossly immature and growth retarded forms, occasional limb hypoplasia and evidence of intrauterine death. The metolazone treatment resulted in a trend toward fewer growth malformations, but this value did not reach statistical significance.

Hematocrit values were as follows: C: 0.52 ± 0.01 ; NP: 0.45 ± 0.02 ; PDS: 0.41 ± 0.02 ; PDSM: 0.41 ± 0.08 . The mean value for the control (C), non-pregnant animals was not statistically significantly different from that for NP ($p > 0.05$), but different from

PDS ($p < 0.05$) and PDSM ($p < 0.05$) groups. However, the latter three treatments did not differ from each other.

Discussion

Definitive therapy of preeclampsia awaits determination of its multiple etiologies and development of measures to directly counteract the pathophysiology of the syndrome. The animal model of preeclampsia used has many of the phenotypic characteristics of human preeclampsia (Outland et al., 2005). Given the effectiveness of metolazone in the therapy of essential hypertension, even when given in non-natriuretic and non-diuretic doses, we elected to examine its use in our model of preeclampsia. We found that the drug was effective in lowering BP to normal pregnant levels without either a natriuresis or the disruption of fluid balance as determined by similar weights in the treated versus untreated animals (see Fig. 2). We did not see resolution of proteinuria and, despite a trend toward improvement in IUGR, the values for pup number and average number of malformations did not reach statistical significance (see Fig. 5A).

However, it may be that the drug was given too late in the pathogenetic process to alter the abnormal glomerular permeability to protein and the vascular abnormalities leading to IUGR. We speculate that if we had instituted metolazone therapy *before* hypertension developed, we might have noted beneficial effects on these parameters.

The current therapy of uncomplicated mild to moderate preeclampsia does not include the use of antihypertensive agents. Once BP rises to levels seen in severe preeclampsia, hydralazine or labetalol is administered to control BP while delivery is planned. Avoidance of treatment of mild to moderate preeclamptic hypertension was practiced in the past because despite improvement in BP, the disease process was thought to continue. Definitive proof that antihypertensive agents used in mild to

moderate preeclampsia as a temporizing measure are harmful has never been well documented.

A general consensus (Lindheimer and Katz, 1973) that diuretics should not be used for treatment or prevention of preeclamptic hypertension and edema exists (Weseley and Douglas, 1962; Krause et al, 1966; Gray, 1968; Pitkin et al., 1972; Christianson and Page, 1976) despite some early reports of successful outcomes with these agents (Cuadros and Tatum, 1964; Finnerty and Bepko, 1966). This concept has resulted from the following observations: 1) Preeclamptic women already have a contracted intravascular volume despite expansion of the interstitial ECF space (Brown et al., 1989); 2) A rat model of pregnancy-induced hypertension developed in the mid-1970s (Abitbol et al., 1976) and 1980s (Losonczy and Mucha, 1989) by reducing uterine perfusion pressure, has been used more recently as a paradigm for the late events of preeclampsia by Granger and coworkers. In this model, hypoperfusion of the maternal-fetal unit led to increased renal vascular resistance and hypertension (Alexander et al., 2001). Accordingly, any additional decrement in ECF volume due to the use of diuretics might be expected to further reduce blood flow to the uterus and exacerbate the pathophysiology of the preeclampsia.

Excessive volume expansion as an important etiologic factor in the pathogenesis of preeclampsia may not be a universal phenomenon in the human syndrome. In our rat model of preeclampsia, we have identified a circulating inhibitor of sodium- potassium ATPase, the excretion of which is elevated *before* hypertension develops (Vu et al., 2005). This substance, marinobufagenin (MBG), could serve, as a predictor of the later development of preeclampsia if the data obtained in rats is applicable to the human

subject. Thus, therapy with metolazone might be attempted only in those patients in whom MBG excretion is elevated.

Past studies of the treatment of preeclamptic hypertension with diuretics have been problematic. The literature is replete with anecdotal reports. There have been no double blind, randomized, controlled investigations with adequate numbers to achieve valid conclusions. Confounding the results of many of these reports is the fact that patients with preeclampsia superimposed upon preexisting hypertension from various etiologies were included. Finally, the doses of the diuretics utilized to treat the hypertension and edema of preeclampsia were natriuretic and, by today's standards, rather large. For example, chlorothiazide was given in doses of 500-1,000 mg/day, equivalent to a dose of hydrochlorothiazide of 50-100 mg/day. These dosages are no longer employed even in the therapy of essential hypertension. Thus, any potentially beneficial effect of this class of drugs on the disease process was most likely vitiated by the introduction of ECF volume contraction. Only in recent years has it been recognized that small doses of these drugs are effective in the therapy of essential hypertension (Puschett, 1999).

IUGR is a common concomitant of preeclampsia (Eskenazi et al., 1993; Obegard et al., 2000; Xiao et al., 2003). In the animal model described herein (Ianosi-Irimie et al., 2005), IUGR occurred. Furthermore, while the values did not reach statistical significance, the data suggest that IUGR might be either prevented or mitigated in this rat model of preeclampsia by the administration of metolazone (Fig. 5).

It is not possible, given our results, to implicate NO as an important pathogenetic factor in these studies. Furthermore, since there were no differences, either in the blood or urine NO levels between the PDS and PDSM rats, it is clear that the mechanism by

which metolazone lowered BP did not involve alterations in NO. These conclusions are verified by the fact that there were no changes either in eNOS or nNOS. We suspect that the increased excretion of NO in the expanded animals was the result of an increment in Na excretion (Shultz and Tolins, 1993) and that the augmented clearance of this substance by the kidney may have led to a decline in the blood levels that were observed.

The mechanism by which metolazone reduced BP is currently unknown. However, since volume contraction was not involved, it must have represented a vasodilatory effect. Because, a NO mechanism is not supported by our data, further studies will be required to determine the nature of this effect.

In conclusion, metolazone has proven effective in ameliorating the hypertension in this rat model of preeclampsia. There was a trend toward a reduction in pup malformations and an improvement in pup number with metolazone that did not reach statistical significance. Perhaps provision of the drug earlier in pregnancy would result in an improvement in these parameters. The latter studies are currently planned.

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Footnotes

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Reprint requests: Gabriella Pridjian, M.D., Department Of Obstetrics & Gynecology, SL-11, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112-2699; e-mail: Gabriella.Pridjian@Tulane.edu.

Legends for Figures

Fig. 1. Mean tail cuff BP (mm Hg) in the four groups of animals. The time t_0 measurements are the baseline readings. t_1 represents the values obtained at 4-7 days of gestation. In the PDSM animals, metolazone administration was then begun. t_2 reflects mean values obtained at 10-13 days of gestation and t_3 measurements were taken at day 17-20, just before sacrifice. Note the statistically significant decrease in BP after treatment with metolazone (PDSM vs. PDS; *** $p < 0.001$).

Fig. 2. Total weight evolution throughout the entire experiment in each group. There were no statistically significant differences in mean weight among the groups at the time periods noted. Except for the control, non-pregnant group ($p > 0.05$), the mean weight in each group increased (day 0 vs. day 18, *** $p < 0.001$, in each case).

Fig. 3. Total urinary sodium excretion in 24-hour increments throughout the experiment. Animals in the PDSM and PDS groups excreted significantly more sodium each day once DOCA and salt were administered when compared to the normal pregnant (NP) and non-pregnant controls (C) (PDSM vs. NP or vs. C and PDS vs. NP or vs. C *** $p < 0.001$). There were no statistically significant differences between the metolazone and DOCA groups and between the normal pregnant and control, non-pregnant groups ($p > 0.05$).

Fig. 4. Mean urinary protein excretion for 24-hours in each group measured at 18-19 days of gestation. Animals in the PDS and PDSM groups displayed significantly greater protein excretion when compared to control and normal pregnant groups (PDSM or PDS vs. C, *** $p < 0.001$; PDSM or PDS vs. NP, †† $p < 0.01$). A numerical increase in urinary protein was also noted in the NP group vs. the control, non-pregnant group,

which did not reach statistical significance ($p > 0.05$). There were no differences, however, between the PDS and PDSM groups.

Fig. 5. A - Blood nitric oxide, as estimated by the determination of nitrite/nitrate (NO_x) in each group on the day before the termination of the experiment. Blood NO_x was significantly higher in all pregnant animals when compared to the non-pregnant control group (NP vs. C, $***p < 0.001$; PDS vs. C, $**p < 0.01$; PDSM vs. C, $*p < 0.05$). PDS and PDSM groups had significantly lower blood NO_x levels than normal pregnant animals (PDS vs. NP, $^{\dagger\dagger}p < 0.01$; PDSM vs. NP, $^{\dagger\dagger\dagger}p < 0.001$). B - Urinary NO_x excretion was higher in the PDS animals when compared to the control and NP groups ($**p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$, respectively). However, despite the numerical trend toward a reduction in urinary NO_x between PDS and PDSM groups, the difference did not reach statistical significance.

Fig. 6. A - Mean pup number in each pregnant group studied. Significantly fewer pups were noted in the PDS group compared to the normal pregnant animals ($*p < 0.05$). B - Mean number of malformations in each group. Severe growth restriction was considered a malformation. The mean values for PDS and PDSM did not differ statistically ($p > 0.05$).

Fig. 1

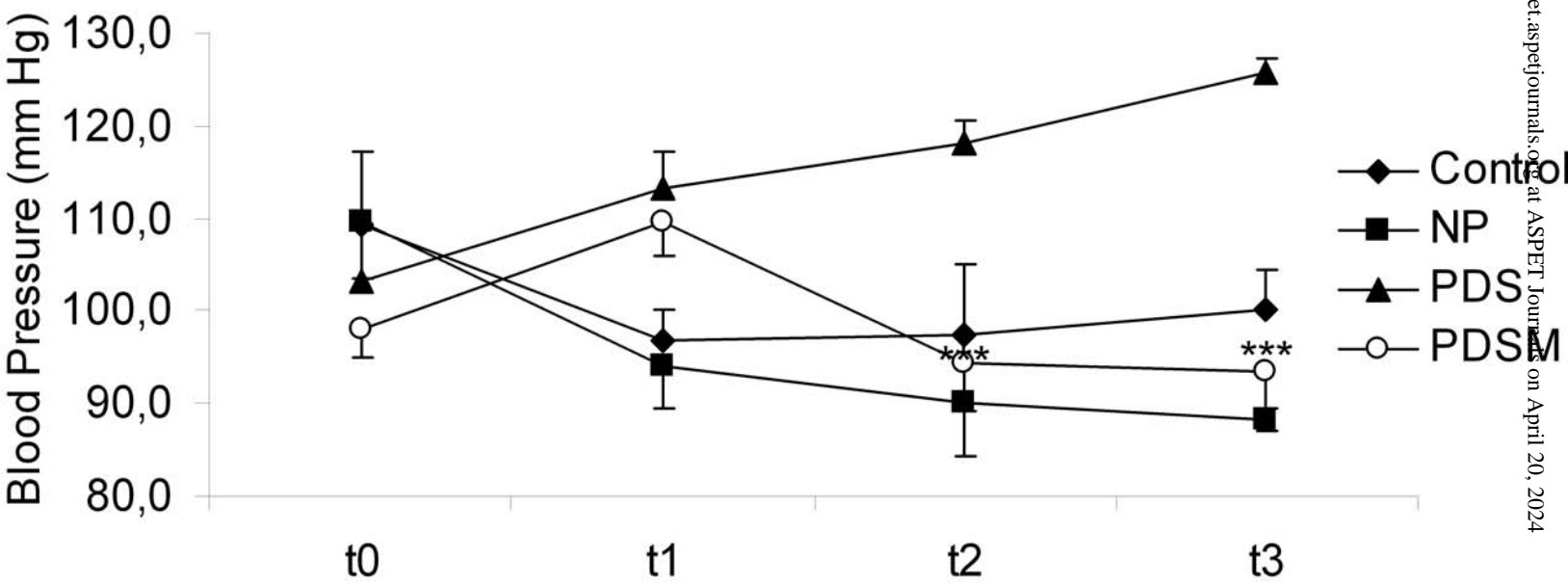


Fig. 2

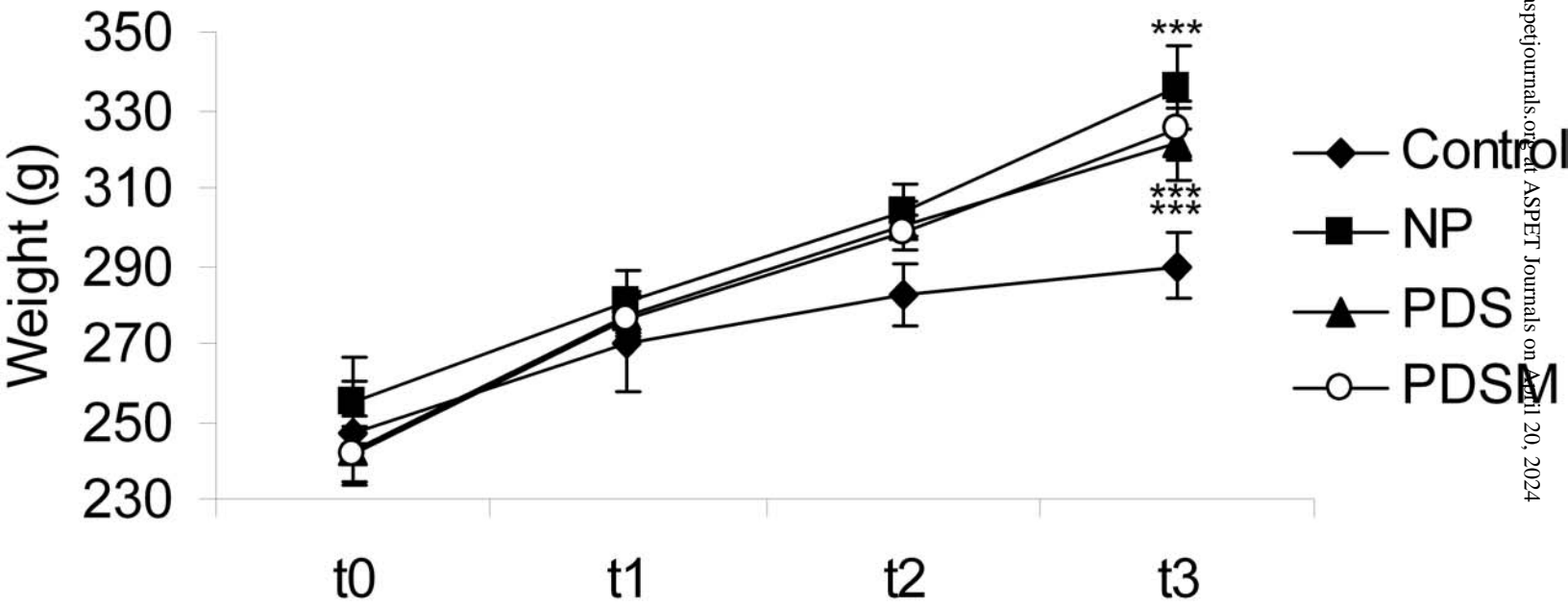


Fig. 3

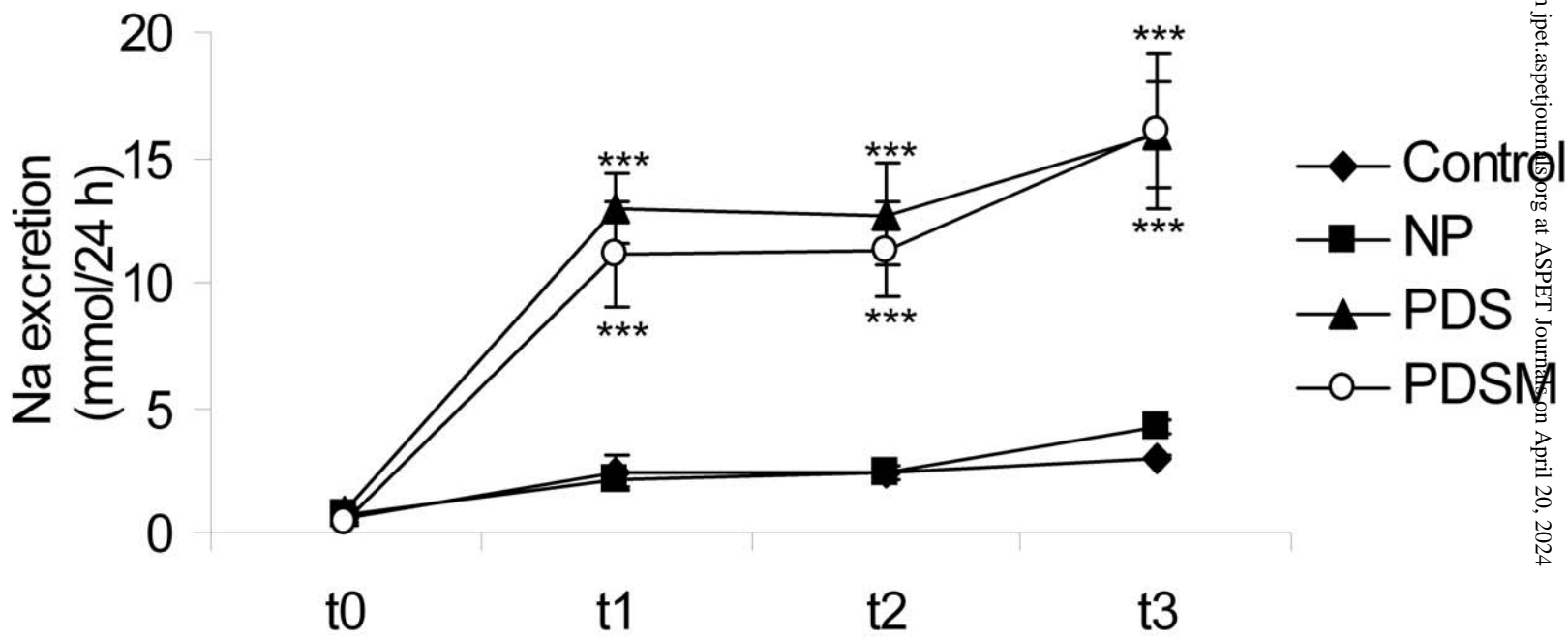


Fig. 4

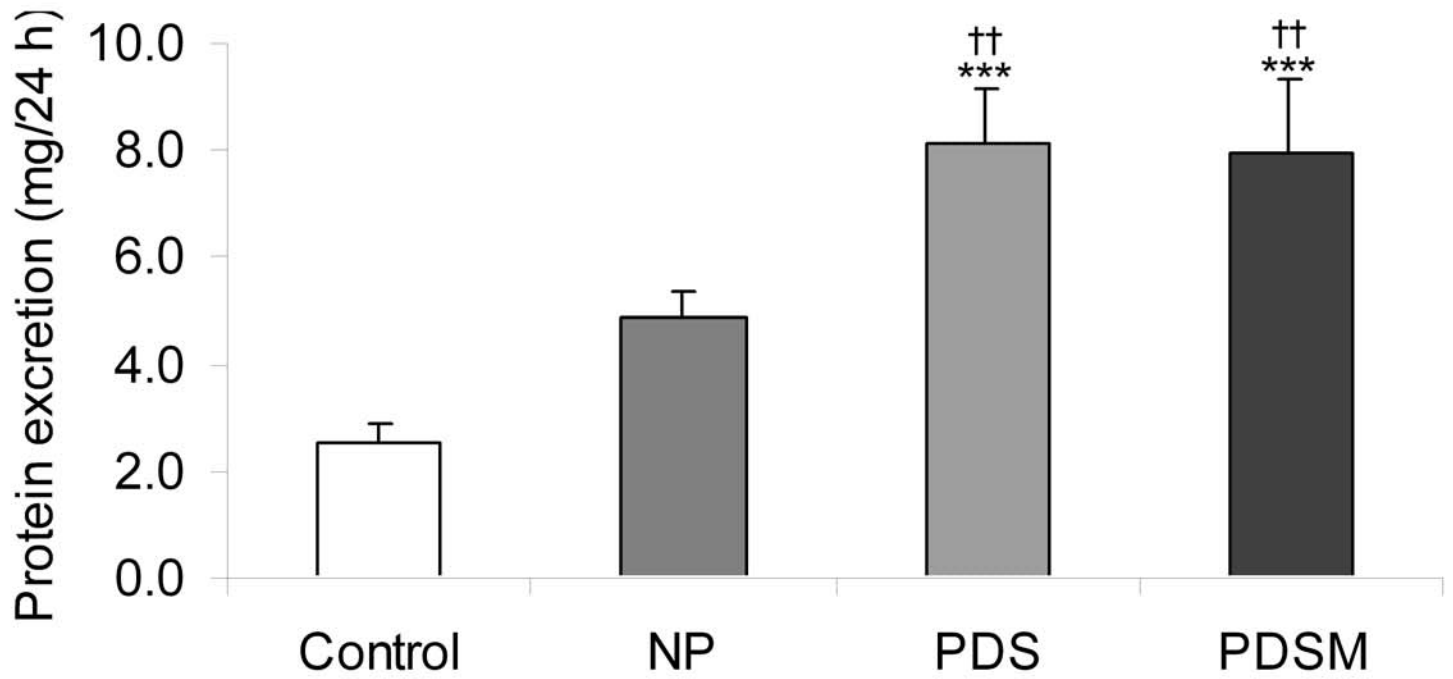


Fig. 5

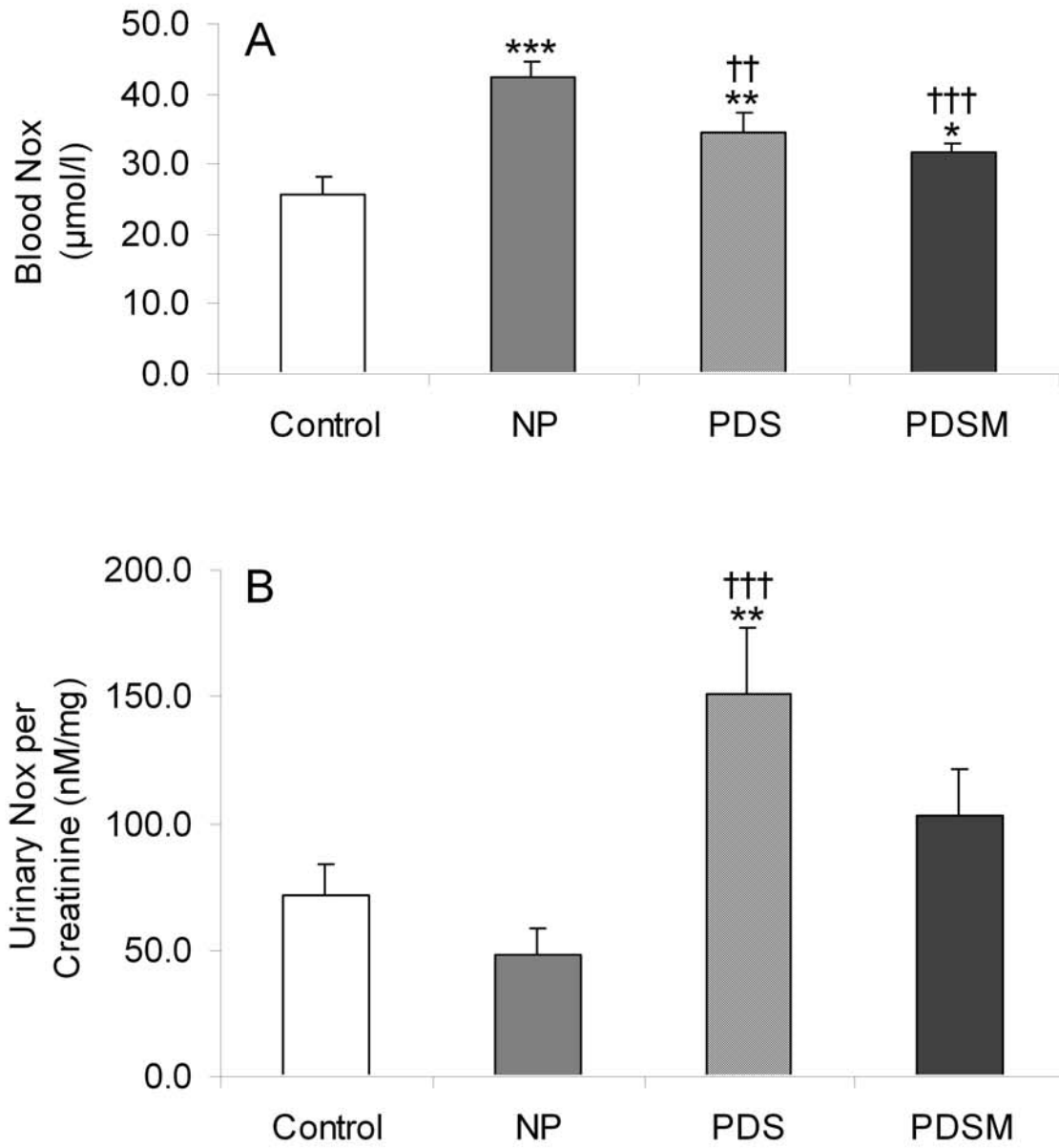


Fig. 6

