Reproductive Dysfunction in Female Rats With Renovascular Hypertension

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BACKGROUND
Hypertension is a major public health epidemic that is highly associated with sexual dysfunction in both men and women. Despite its high prevalence, clinical and animal literature on the underlying mechanisms of sexual dysfunction in hypertensive women is remarkably limited.

METHODS
Using a well-established rodent model of renovascular hypertension—the 2-kidney, 1-clip (2K1C) Goldblatt model—we investigated possible reproductive deficits in female rats. We evaluated several aspects of reproductive function in hypertensive female rats: estrous cycle, sexual behavior, ovulation, and plasma levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol at proestrus afternoon.

RESULTS
Clipping of the left renal artery resulted in dramatic elevations in systolic blood pressure and heart rate. Renovascular hypertension was associated with a delay for reestablishing estrous cyclicity (50% of 2K1C rats failed to resume cycling by 15 days after surgery). In rats that resumed cycling, 2K1C female rats showed a decrease in sexual behavior, evidenced by a decreased lordosis quotient and a reduction in ovulation, as demonstrated by a decreased number of oocytes. Moreover, plasma levels of LH on the proestrus afternoon were reduced in hypertensive female rats, but no changes in estradiol or FSH were observed.

CONCLUSIONS
Our results demonstrate that renovascular hypertension induces an overall decrease in reproductive function in female rats. Most important, our results indicate that the animal model of renovascular hypertension could be used as a relevant tool to understand better the pathophysiological mechanisms involved in the reproductive deficits in women with renovascular hypertension.

Keywords: blood pressure; estrous cycle; female rats; hypertension; ovulation; renovascular hypertension; reproduction; sexual behavior.

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Hypertension affects >25% of the general adult population in Western countries and is considered a major public health epidemic,1 especially because of its comorbidity with cardiovascular and kidney diseases. In addition to the myriad health complications associated with hypertension, significant evidence suggests that hypertension and/or its treatment are highly correlated with sexual dysfunction in both men and women.2–5 Specifically, hypertensive men are more likely than normotensive men to suffer erectile dysfunction.4 Similarly, hypertensive women experience decreased vaginal lubrication, less frequent orgasm, and more frequent genital pain in comparison with normotensive women.2,7 Renovascular hypertension represents the most frequent secondary form of arterial hypertension, with prevalence ranging from 3%–5% in the general hypertensive population and up to 45% in accelerated and malignant hypertension.3

However, clinical and preclinical studies have given little to no attention to sexual dysfunction in individuals with renovascular hypertension. Indeed, clinical reports do not discuss the prevalence of sexual dysfunction in renovascular hypertension patients. In the animal literature, only a few studies using the well-established rodent model of renovascular hypertension—the 2-kidney, 1-clip (2K1C) Goldblatt model9,10—have analyzed reproductive function in male rats, reporting a drastic impairment in reproductive functions.11,12 Clinical data support the suggestion that sexual dysfunction is more frequent in women than in men.13,14 Despite this higher prevalence, clinical and preclinical studies on female sexual dysfunction are remarkably scarce, and the mechanisms by which hypertension interferes with female reproductive function are still not fully understood.15 Here, we used the 2K1C renovascular hypertension model9,10 to investigate possible reproductive deficits in female rats. The establishment and maintenance of hypertension in the 2K1C model depend mainly on an increase in the activity of the renin–angiotensin system.16,17 Kidney underperfusion,
induced by clamping the renal artery, leads to increased levels of renin and angiotensin II (Ang II), which induce, among other effects, a sustained increase in blood pressure.

Besides its "classic" role in modulating fluid balance and cardiovascular function, Ang II also participates in the regulation of normal reproductive function. Central levels of Ang II increase during the proestrus afternoon preceding the onset of the luteinizing hormone (LH) surge. Indeed, intracerebroventricular infusion of Ang II induces an increase in LH secretion during the proestrus afternoon or in ovariec-tomized rats primed with estradiol and progesterone.

Ang II's important role in female reproductive physiology suggests that the 2K1C model of hypertension, which induces increases in Ang II, is a relevant animal model for evaluating reproductive deficits affecting women with renovascular hypertension. In this study, we evaluated the estrous cycle, sexual behavior, ovulation, and sexual hormones during the proestrus afternoon of female rats submitted to the 2K1C model of hypertension. Here, our aim was to establish an animal model that could be used to understand better the underlying mechanisms involved in reproductive dysfunction in women with renovascular hypertension.

METHodS
Animals
Female Wistar rats (aged 90 days) were brought from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) to the animal room in our laboratory. The room was maintained at a constant temperature (22 ± 1 °C) and on a 12-hour light–dark cycle (lights on at 6 am). Rats were given ad libitum access to water and food (rodent chow, Nutrilab, Colombo, Brazil). Experiments were performed in accordance with National Institutes of Health and Brazilian College of Animal Research guidelines and approved by the University Research Committee of the Federal University of Rio Grande do Sul.

Experimental design
Estrous cycles were checked daily before and after surgery (2K1C or sham) in all rats, continuing until the last day of the experiment. Only rats with 3 consecutive and regular 4-day estrous cycles were used for 2K1C or sham surgery. The systolic blood pressure (SBP) and heart rate of all rats were measured approximately 10 days after surgery on the diestrus day. Two or 3 estrous cycles after the SBP measurement, all rats that resumed estrous cyclicity were used for sexual behavior testing (cohort 1: proestrus night) or for blood sampling (cohort 2: proestrus afternoon). The following day (estrus morning), both cohorts of rats (sexual behavior testing or blood sampling) were decapitated for assessment of ovulation, clip placement, and kidney weight.

2K1C surgery
Rats’ estrous cycles were monitored by daily vaginal smear analysis at 9:00 AM. Only female rats in diestrus that had displayed at least 3 consecutive 4-day estrous cycles were used for surgery. Surgical procedures were performed under ketamine (Ketalar; Parke-Davis, São Paulo, Brazil; 100 mg/kg intraperitoneally) and xylazine (Rompun; Bayer, São Paulo, Brazil; 50 mg/kg intraperitoneally) anesthesia. A silver clip (0.15-mm internal diameter) was placed around the left renal artery though a flank incision. The same surgical procedure was used for sham-operated rats, except for the placement of the renal artery clip.

Estrous cycle
After surgery, estrous cycle was verified daily for a maximum of 30 days by analyzing fresh vaginal smears taken at 9:00 AM under optic microscope. Analysis of the estrous cycle consisted of calculating the percentage of rats showing regular estrous cycling 15 days after surgery. From rats that resumed regular estrous cycling, we recorded the number of days they took to resume. Only rats that resumed estrous cyclicity after surgery were used for sexual behavior testing, blood sampling, and ovulation analyses.

Systolic blood pressure
Sham and 2K1C rats had their SBP and heart rate (bm⁻¹) measured approximately 10 days after surgery on the diestrus day, by a noninvasive tail-cuff method using the RTPBP1001 Rat Tail Blood Pressure System (Kent Scientific Corp, Litchfield, CT). Prior to recording, rats were acclimated for 3 consecutive days for 1 hour in the testing apparatus. The rat was kept in an acrylic tube with the tail free for placement of the sphygmomanometer and blood pressure transducer for arterial pressure recording. SBP was recorded for 5 minutes, and at least 5 consecutive pressure measurements were taken for each rat, with the SBP for each rat calculated as the average. Only 2K1C rats with mean SBPs of >150 mm Hg were included in this study.

Sexual behavior
Following SBP determination and completion of 2 or 3 regular estrous cycles, sexual behavior was recorded under red light during the first 2 hours after the beginning of the dark cycle of the proestrus night. Testing was performed using a proven breeder male rat in an observation cage (70 cm long × 70 cm wide × 35 cm high). Rats were videotaped for 15 minutes, and the total number of lordosis displays by the female rat and the number of mounts and intromissions by the male rat were scored. The lordosis quotient, an index of female sexual receptiveness, was calculated by dividing the number of lordosis displays by the number of mounts with or without intromissions. The frequency of locomotion and the percentage of time mobile were also scored to rule out any locomotor deficits in hypertensive female rats during the sexual behavior test.

Jugular cannulation and blood samples
On the morning of the second or third proestrus after SBP determination, between 11:00 AM and 12:00 PM, sham
and 2K1C rats were submitted to jugular vein cannulation. Rats were anesthetized with tribromoethanol (Aldrich; 1 mL of a 2.5% solution/100g body weight intraperitoneally), and a silastic catheter was inserted through the external jugular vein into the right atrium. At 12:30 pm, polyethylene tubing (PE-50) was connected to the jugular catheter and filled with saline (0.9%), and the rats remained undisturbed in their cages for an additional 30 minutes until the beginning of the experiment at 1:00 pm. Blood samples (600 µL) were collected in plastic heparinzed syringes every hour (1:00 pm–8:00 pm) on the proestrus day. After each blood sample was taken, 600 µL of 0.9% sodium chloride was injected to replace the volume removed. Plasma was separated by centrifugation (4ºC) and stored at −80ºC until assay.

**radioimmunoassay**

Plasma estradiol concentrations were determined by double-antibody radioimmunoassay using specific Magnetic Antibody Immunoassay (MAIA) kits (BioChem ImmunoSystems, Bologna, Italy). The lower limit of detection for estradiol was 7.5 pg/ml. Radioimmunoassays for LH and follicle-stimulating hormone (FSH) were performed using specific kits provided by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Los Angeles, CA). The antibodies used were antirat LH-S10 and FSH-S11; the standards were LH-RP3 and FSH-RP2. The lowest limit for detection was 0.05 ng/ml for LH and 0.2 ng/ml for FSH. For each hormone radioimmunoassay, all samples were measured in the same assay. The intra-assay coefficients of variation were 2.5% for estradiol, 4% for LH, and 3% for FSH.

**ovulation**

On the morning (9:00 AM) of the estrus day and following sexual behavior testing on proestrus night or blood sampling on proestrus afternoon, female rats were decapitated, their ovaries were removed, and the oviducts were dissected and pressed between 2 microscope slides. The number of oocytes of both oviduct ampullae was counted under the microscope (Zeiss, Goettingen, Germany) with a ×2.5 lens.

**Body and kidney weights and renal index**

At the end of the experiment, rats were weighed and sacrificed to allow for clip placement verification. Clipped and contralateral kidneys were weighed, and the renal index was calculated by dividing the kidney weight (mg) by the body weight (g).

**Statistical analysis**

All data show a normal distribution and were expressed as means ± SEMs. Sexual behavior, ovulation, the amount of time taken to reestablish regular estrous cycling, and the areas under the curve for plasma LH, FSH, and estradiol were analyzed using Student’s t test. The number of rats that resumed regular estrous cycling was analyzed using χ² with the data expressed in percentages. Plasma levels of LH, FSH, and estradiol were analyzed using a repeated measure 1-way analysis of variance (within groups and time) followed by the Newman–Keuls test for multiple comparisons. In all cases, statistical significance was set at P < 0.05.

**RESULTS**

**Establishment of the renovascular hypertension in female rats**

As shown in Table 1, hypertension induced by clipping the left renal artery did not induce changes in body weight (t(52) = 1.55). However SBP (t(52) = 18.69; P < 0.0001) and heart rate (t(52) = 5.39; P < 0.0001) were significantly higher in 2K1C female rats than in sham rats. The right kidney weight was significantly increased (t(52) = 6.74; P < 0.0001) and the left kidney weight was significantly decreased (t(52) = 12.73; P < 0.0001) in hypertensive female rats when compared with sham female rats. These changes in kidney weight resulted in alterations in the renal index that were significantly higher for the right kidney (t(52) = 7.96; P < 0.0001) and lower for the left kidney (t(52) = 11.71; P < 0.0001) in the hypertensive group when compared with sham rats.
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**Figure 1.** Hypertension dysregulated estrous cyclicity. (a) Only 50% of 2-kidney, 1-clip (2K1C) rats resumed regular estrous cycling by the 15th day after surgery, compared with 100% of sham rats who resumed regular estrous cycling within the same time frame (n = 26 for sham rats, n = 56 for 2K1C rats). (b) When considering only those females that resumed estrous cyclicity, the hypertensive females were delayed in resuming regular estrous cycling following surgery (n = 26 for sham rats, n = 28 for 2K1C rats). *P < 0.0001, significant difference from control.

**Figure 2.** Hypertension induced a reduction in number of oocytes in the morning of estrus. *P < 0.0001, significant difference from control (n = 26 for sham rats, n = 28 for 2-kidney, 1-clip (2K1C) rats).

**Estrous cycle**

Fifteen days after surgery, only 50% of 2K1C female rats had resumed regular estrous cycling, whereas 100% of sham rats showed regular estrous cycling (χ²(1, N = 82) = 19.74; P < 0.0001) (Figure 1a). When analyzing the rats that resumed regular estrous cycling, we observed that hypertensive female rats showed a significant delay (t(52) = 6.89; P < 0.0001) reestablishing regular estrous cycling after the surgery when compared with sham rats (Figure 1b).

**Ovulation**

Hypertensive rats exhibited a significant reduction in the number of oocytes on the estrus morning when compared with sham rats (t(52) = 6.83; P < 0.0001) (Figure 2).

**Sexual behavior**

Renovascular hypertension induced a reduction in sexual receptivity. Specifically, 2K1C female rats presented a significant reduction in lordosis (t(30) = 2.75; P < 0.01) (Figure 3a) when compared with sham rats. However, the number of mounts with or without intromission performed by the male rats used to test the hypertensive or sham female rats was not different between the 2 groups (t(30) = 0.79) (Figure 3b), which resulted in a significant reduction in the lordosis quotient in 2K1C female rats (t(30) = 3.30; P < 0.002) (Figure 3c). The reduction in sexual behavior in hypertensive rats was not due to deficits in locomotion. The frequency of locomotion (t(30) = 0.96) (Figure 3d) and the percentage of time mobile (t(30) = 0.47) (Figure 3e) were not different between 2K1C and sham female rats.

**Plasma levels of LH, FSH, and estradiol on the afternoon of proestrus**

Plasma levels of LH were significantly lower in hypertensive rats during the proestrus afternoon (Figure 4a). Analysis of variance revealed a significant interaction between group and time (F(7,20) = 5.90; P < 0.0001) and a main effect of time (F(7,20) = 24.80; P < 0.0001), but no significant main effects for group (F(1,20) = 0.80). Post hoc analysis for the interaction between group and time revealed a significant reduction in LH plasma levels at 4:00 PM in 2K1C rats when compared with sham rats. The area under the curve of LH in 2K1C female rats was significantly lower than in controls (t(20) = 2.28; P < 0.04) (Figure 4b).

FSH levels during the proestrus afternoon were not different between hypertensive and normotensive female rats (Figure 4c). Analysis of variance revealed only a significant main effect of time (F(7,21) = 22.16; P < 0.0001). No significant main effects of group (F(1,21) = 0.59) or interaction between group and time (F(7,21) = 0.35) were detected. Post hoc analysis for the main effect of time shows an FSH peak from 4:00 PM to 8:00 PM, independent of group. The areas under the curve of FSH were similar between groups (t(21) = 0.99) (Figure 4d).

Estradiol levels during the proestrus afternoon were not different between hypertensive and normotensive rats (Figure 4e). Analysis of variance revealed only a significant main effect of time (F(7,21) = 17.54; P < 0.0001). No significant main effects of group (F(1,21) = 0.20) or interaction between group and time (F(7,21) = 1.83) were detected. Post hoc analysis for the main effect of time shows that estradiol plasma concentrations from 1:00 PM until 5:00 PM were higher than concentrations observed during 6:00 PM until 8:00 PM, independent of group. The areas under the curve of estradiol were similar between groups (t(21) = 1.49) (Figure 4f).

**DISCUSSION**

Clinical reports suggest a strong association between hypertension and/or its treatment and sexual dysfunction in men and women.2–7 The clinical literature is still unclear, however, as to the exact cause of sexual dysfunction in hypertensive patients; that is, it could be a direct result of hypertension or a secondary effect of its treatment.25 Regardless of this issue, relatively few studies from the preclinical literature have addressed the connection between hypertension and sexual dysfunction in female rats. The results of these preclinical studies indicate that spontaneously hypertensive female rats show reduced ovulation26 and morphological changes in the clitoris.15 Even less attention has been given...
to sexual dysfunction that may affect subjects with renovascular hypertension. Confirming studies in male rats,11,12 the data presented here indicate that renovascular hypertension promotes an overall reduction in female reproductive function in rats. Specifically, 2K1C female rats show decreased oocyte numbers, sexual behavior, and LH levels during the proestrus afternoon when compared with control sham rats.

Hypertension and estrous cycle

Our results indicate that 2K1C female rats were significantly delayed in resuming postsurgical regular estrous cyclicity. Most important, 50% of hypertensive female rats did not resume estrous cyclicity in the 30 days analyzed and consequently were not used in the remaining experiments. Among the 50% of 2K1C rats that did resume estrous cyclicity, the estrous cycles were not as regular as in sham rats. In fact, several of the 2K1C rats presented a longer estrous cycle, often remaining in diestrus for a second or third day. To our knowledge, only 1 study using 2K1C hypertensive rats has analyzed estrous cyclicity and suggests a similar distribution of cycle stages between 2K1C hypertensive and normotensive female rats.27 However, this assumption was based on the collection of the vaginal smears only at the completion of the study and based on the observation that all stages of the estrous cycle were represented at that point. Because of our daily checks of estrous cyclicity, we believe that the data presented here provide a more comprehensive look at estrous cycling in the 2K1C model.

Hypertension and sexual behavior

Sexual behavior results presented here confirm clinical studies2–4 demonstrating that renovascular hypertension induces a significant reduction in female sexual receptivity. This reduction in sexual receptivity in 2K1C rats was not due to motor impairments because locomotion was not altered in hypertensive rats when compared with sham rats. Moreover, our results also corroborate other animal studies in which renovascular hypertensive male rats have shown sexual dysfunction.11,12,28

The estradiol surge in proestrus is a required step to induce lordotic behavior.29 However, 2K1C rats did not show any changes in estradiol levels in the proestrus afternoon, suggesting that the decreased sexual receptivity presented here is supported by other mechanisms. One possible mechanism might involve Ang II because central Ang II is known to play an inhibitory role on sexual behavior.30 Specifically, infusion of Ang II into the medial amygdala decreases sexual behavior in female31 and male32 rats. Furthermore, Morishita and colleagues33 have shown that, in male rats, renovascular hypertension induces a significant increase in central levels of Ang II. We suggest that the reduction in sexual receptivity in hypertensive female rats presented here could be due to an increase in central levels of Ang II, which have an inhibitory role in the neurocircuitry underlying sexual behavior. Future studies using the current model will be important in further delineating Ang II’s role in suppressing sexual behavior in hypertensive rats.

Moreover, in hypertensive female rats, the possible attenuating effects of increased systemic blood pressure on lordotic

Figure 3. Hypertension decreased female sexual behavior in the night of proestrus. (a) Two-kidney, 1-clip (2K1C) rats showed a reduction in lordotic behavior. However, the number of mounts performed by the males was not different (b), resulting in a reduction in the lordosis quotient in 2K1C rats (c). Frequency of locomotion (d) and percentage of time in locomotion (e) was not different between groups. *P < 0.01, significant difference from control (n = 16 for both groups).
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behavior cannot be discarded. The periaqueductal gray area is known to play an important role in autonomic regulation, including blood pressure and heart rate34,35 and is also a major site supporting sexual behaviors such as lordosis34,36. These periaqueductal gray functions are not isolated34 and the interaction between these different functional components of the periaqueductal gray may play a role in the reduced sexual behavior observed in our hypertensive female rats.

Hypertension and ovulation

Renovascular hypertensive rats showed decreased ovulation, as demonstrated by 2K1C female rats displaying reduced oocyte numbers when compared with sham rats.

This result confirms previous data in spontaneously hypertensive female rats, where the number of oocytes was lower than that found in controls.26 Ovulation is a dynamic and complex process that is triggered by a sharp surge of several hormones in the preovulatory period, including steroid hormones, LH, and FSH. The LH surge in the proestrus afternoon is one of the key events in the promotion of ovulation.37,38 Our results show that hypertensive rats had a significant reduction in the LH surge during the proestrus afternoon, which may be implicated in the ovulatory reduction of 2K1C female rats. It is important to consider, despite the oocyte reductions observed in 2K1C female rats, that the FSH total secretion in the proestrus afternoon was not different between groups.

Another factor that could contribute to the decreased ovulation in hypertensive rats is a possible reduction in blood flow to the ovary. Increases in blood flow to the follicular cells in the preovulatory follicle wall are necessary for providing the required nutrient supply, hormonal substrates, and other blood components essential for ovulation.39 This essential increase in ovarian blood flow could be compromised in hypertensive individuals, especially in those with renovascular hypertension, which is characterized by an increase in peripheral Ang II levels. As a potent vasoconstrictor, Ang II could be acting on ovarian arteries to decrease ovarian blood flow and cause a subsequent reduction in oocyte numbers in our hypertensive female rats. Supporting this notion is the fact that increased expression of Ang II in hypertensive transgenic (mRen-2)27 rats or systemic infusion of Ang II in normal rats results in a reduction in oocyte numbers when compared with controls.40

Implications

Although the clinical literature has consistently reported hypertensive-associated dysfunctions in sexual behavior, the analysis of reproductive function in 2K1C female rats presented here reveals a complex scenario that includes overall reproductive deficits. This suggests that the renovascular hypertension model, developed by Goldblatt,9 can be a valuable tool in better understanding the underlying pathophysiologic mechanisms of sexual dysfunction in renovascular hypertensive women.

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DISCLOSURE

The authors declared no conflicts of interest.
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