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Treatment of spontaneously hypertensive rats during pregnancy and lactation with the antioxidant tempol lowers blood pressure and reduces oxidative stress

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Abstract: Genetic and environmental factors interact in a complex manner in the pathogenesis of essential hypertension in humans. Oxidative stress is considered one of the more important environmental factors. We used the spontaneously hypertensive rat (SHR) model to test whether continuous feeding with the antioxidant tempol reduces maternal oxidative stress during pregnancy and potentially contributes to the prevention of cardiovascular disease onset. Pregnant female rats were divided into control and tempol-treated groups. Tempol was continuously administered in drinking water. The administration period lasted approximately 40 days, from the confirmation of a vaginal plug until birth of the pups and their subsequent weaning. The blood pressure (BP) of each adult female was measured three times during pregnancy and post parturition. Milk was collected three times from nursing mother rats in the immediate postpartum period. Markers of oxidative stress were measured: 8-hydroxyl-2′-deoxyguanosine (8-OHdG) levels in milk during the experimental period and 8-OHdG and corticosterone levels in urine of adult and neonatal rats. The urinary level of 8-OHdG in the tempol-treated group was significantly lower than that in the control group. Corticosterone levels were significantly lower in urine of neonatal rats from the tempol-treated group compared with the levels of the control group. The levels of total antioxidant in milk were significantly greater in the tempol-treated group than in the control group. This study demonstrated that continuous administration of tempol to pregnant SHRs reduced maternal oxidative stress and contributed to reduced oxidative stress in neonatal rats.
Key words: antioxidant, oxidative stress, pregnancy, spontaneously hypertensive rat (SHR), tempol

Introduction

Currently, one-third of human adults have high blood pressure (BP), otherwise known as hypertension [1]. The Guidelines for the Management of Hypertension 2019 (JSH2019) in Japan indicate that approximately 43,000,000 adults are hypertensive. Moreover, management of hypertension is reported to be poor in 31,000,000 patients with a BP above 140/90 mmHg [2]. Despite recent advances in methods for treating hypertension, elevated BP is still a primary etiological factor in morbidity [3]. Hypertension arises through interactions between genetic and environmental factors. It has been shown that it is important to improve lifestyle-related factors, including diet, to prevent onset of hypertension and to reduce hypertension in severe cases. Increased oxidative stress in particular can induce vascular endothelial damage through inactivation of nitric oxide (NO), contributing to the onset of hypertension [4]. Oxidative stress refers to an imbalance between the production of reactive oxygen species (ROS) and free radicals and the antioxidant defense system involving antioxidant en-

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zymes; such an imbalance can lead to an oxidation-predominant tendency [5, 6]. In addition, excessive generation of ROS can induce DNA damage, lipid peroxidation, and protein degeneration; an excess of ROS may function as an etiological/exacerbation factor for various diseases, such as cancer, and aging [7]. Excessive generation of ROS is believed to be involved in the onset or progression of lifestyle-related diseases and metabolic syndrome [8, 9].

It has been suggested that one of the reasons why the incidence of hypertension continues to increase may be related to childhood development factors [10–12]. Furthermore, a theory, termed the "Developmental Origins of Health and Disease (DOHaD)," was proposed which speculated that predictive adaptive responses might occur in response to the environment during the early developmental process (fetal period and immediately postnatal) [13]. With regard to human obstetrics, changes in lifestyle, such as an increase in late childbearing, a western diet, and lack of exercise, may increase the rate of hypertension among pregnant women; additionally, increased oxidative stress might be involved in disease onset or progression [14–16]. It is known that risk factors, such as proteinuria and kidney disease, contribute to the progression of hypertensive disorders during pregnancy [17]. Chronic oxidative stress has also been associated with reproductive dysfunction or infertility in humans, while ovarian aging in women may be related to the accumulation of oxidative stress effects [18, 19]. Disturbance to arterial blood flow due to oxidative stress may inhibit fetal growth [20]. Thus, there are several lines of evidence linking the health of the intrauterine environment to subsequent health after birth [21, 22].

Although there has been a number of studies examining the effects of antioxidants on hypertension in the spontaneously hypertensive rat (SHR) model [23–26], the influence of maternal oxidative stress during pregnancy and parturition and its effect on neonatal development have not been investigated. Here, we quantified maternal oxidative stress in pregnant SHRs with and without treatment with the antioxidant compound tempol; additionally, we quantified the total antioxidant concentration in milk and evaluated the neonatal rat urine levels of corticosterone at 21 days post parturition.

Materials and Methods

Animals and breeding conditions

In this experiment, we used 10-week-old male/female SHR/Izm rats (Disease Model Cooperative Research Association, Kyoto, Japan). The following housing conditions were employed: temperature, 23 ± 2°C; humidity, 55 ± 10%; frequency of ventilation, 10 to 13 cycles/h; lighting cycle, 12 h light (7:00–19:00)/12 h dark (19:00–7:00). The rats were acclimated to TPX cages (W260 × D330 × H170 mm, Natsume Seisakusho Co., Ltd., Toyonaka, Japan) containing wood chips (Clean Chip, SHIMIZU Laboratory Supplies Co., Ltd., Kyoto, Japan). During the acclimation period, a standard diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were given. This experiment was conducted according to the Shimane University guidelines for animal experiments under approval by the animal experiment special committee (approval no. IZ30-94).

Experimental methods

An outline of the experimental protocol is shown in Fig. 1. Female rats were mated 1:1 with male rats. The females were separated into two groups the day after the confirmation of a vaginal plug: a control group given only distilled water (n=8) and a group that received the antioxidant tempol (3 mmol/l; 4-Hydroxy-TEMPO, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) in drinking water (n=8). The concentration of the test drug was based on literature that described changes in BP after tempol treatment [27, 28]. Standard food was given *ad libitum*. In the tempol-treated group, the concentration was adjusted every day, and the distilled water/tempol was replaced daily. The rats were given tempol for 40 days from the day after the confirmation of a vaginal plug until delivery or weaning. The number of neonatal rats per mother was adjusted to 6 to 8 at the time of birth so that the lactation volume after parturition would be essentially uniform. Surplus neonatal rats (≥8 in a litter) were sacrificed using carbon dioxide.

Measurement items

Before pregnancy, during pregnancy (days 7 and 14), and during lactation (days 7, 14, and 21 after parturition), body weight and BP were measured. For BP, systolic BP (SBP) and diastolic BP (DBP) were measured using a noninvasive BP meter (BP-98A, Softron Tokyo, Japan). For these measurements, the rats were first placed in an incubator (38°C, 8 min) for warming, and measurement was conducted 5 times using the tail-cuff technique. The mean value was regarded as the BP measurement. To determine whether treatment of the pregnant rats with tempol affected fetal development, we compared the numbers and sex ratios of the offspring in the two groups.

Milking was performed at 1, 2, and 3 weeks after delivery, and the milk volume was measured. The protocol for milking was as follows: (1) the rats were anesthetized using isoflurane (Mylan EPD G.K., Tokyo, Japan) via a simple inhalation anesthesia system for small animals

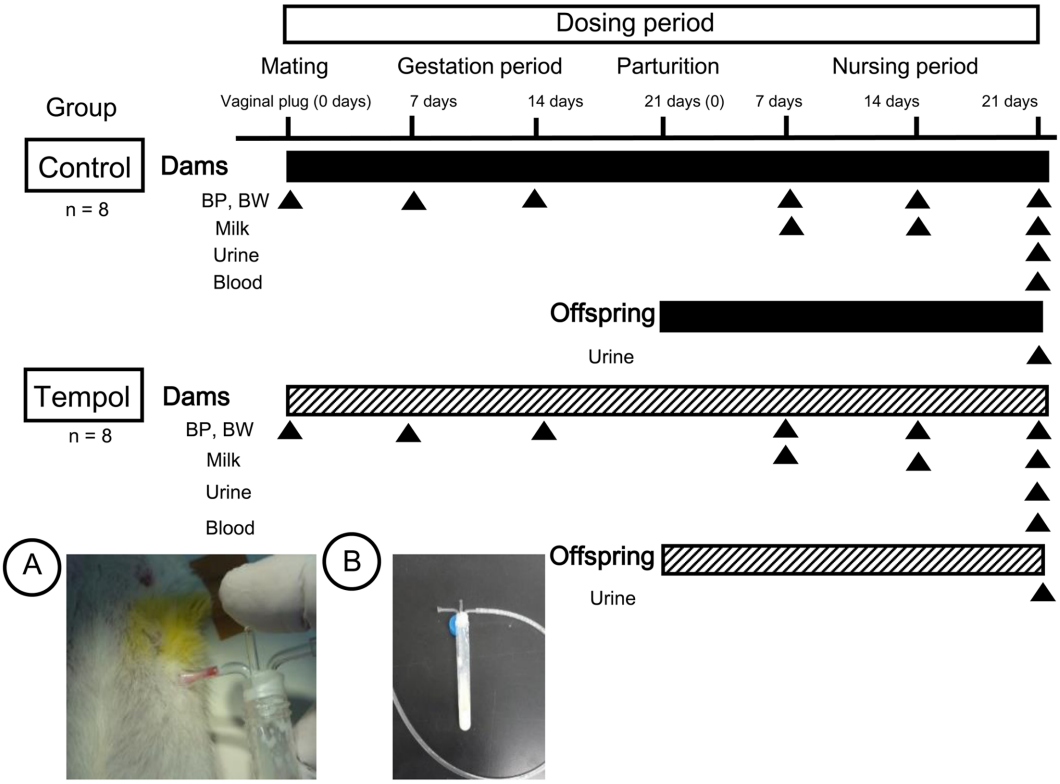

Fig. 1. Experimental protocol. Female rats were separated into two groups: a control group given only distilled water and a group that received the antioxidant tempol in drinking water. The rats were given tempol for 40 days from the day after the confirmation of a vaginal plug until the pups were weaned. Body weight (BW) and blood pressure (BP) were measured on the days indicated by arrows. Milk, urine, and blood were collected during the experimental period. (A) An image of the one-handed milking device for rats; this device was sequentially attached to each of the 12 nipples of the rats to obtain milk. (B) Milking was conducted for a total of 15 min, while adjusting the sucking force of the vacuum pump.

(NARCOBIT-E, Natsume Seisakusho Co., Ltd., Tokyo, Japan): (2) Oxytocin (0.1 unit/kg, Sumitomo Pharma Animal Health Co., Ltd., Osaka, Japan) was then subcutaneously administered to the dorsal region of the rats 10 min before milking under anesthesia. Oxytocin is a natural hormone that stimulates uterine contractions in parturition and lactation; it stimulates contraction of breast tissue to aid in lactation after parturition [29]. (3) The milking pipe of a one-handed milking device for rats (Natsume Seisakusho Co., Ltd., Tokyo, Japan) was sequentially attached to each of the rat's 12 nipples, and milking was carried out for a total of 15 min while adjusting the sucking force of the vacuum pump [30] (Fig. 1A). (4) Milk was collected in sample bottles and stored at −80°C (Fig. 1B).

Urine was collected from neonatal rats at 21 days post birth using the single sampling method. Briefly, the rats were held in the left hand from the dorsal side, and the lower abdomen was slowly rubbed against the urinary meatus with the right hand to collect urine in a sample tube. Samples were stored at −80°C until analysis. At 21 days post parturition, 24 h urine collection was carried out for each adult female by using a metabolic cage (CT-10S type II, CLEA Japan, Inc., Tokyo, Japan). Rat blood was collected from the abdominal inferior vena cava under anesthesia with isoflurane. Collected blood was transferred to a micro blood-sampling tube (Capiject, ethylenediaminetetraacetic acid (EDTA)-2Na, Terumo Corp., Tokyo, Japan) and centrifuged at 860 × g for 15 min at 4°C, and the supernatant was collected as a plasma sample. Each sample was centrifuged and stored at −80°C until analysis.

Determination of oxidative stress and antioxidative activity

Oxidative stress was quantified by measuring the levels of 8-hydroxyl-2′-deoxyguanosine (8-OHdG) in the urine of adult females with a New 8-OHdG Check kit (Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd., Shizuoka, Japan) at 21 days post parturition. Furthermore, after ultrafiltration of the rat milk (Nanosep 10KDa centrifugal filter, Nihon Pall Ltd., Tokyo, Japan), the filtrate was analyzed using a highly sensitive method for assessing 8-OHdG (Highly Sensitive 8-OHdG Check

ELISA kit, Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd.). As stressful stimuli activate the hypothalamo-pituitary-adrenocortical axis, we measured the level of corticotropin releasing factor (CRF) in the plasma with a Mouse/Rat CRF-HS ELISA kit (Yanaihara. Co., Ltd., Shizuoka, Japan) according to the manufacturer's protocol. Adult and neonatal rat urine levels of corticosterone (ARK Checker CORT EIA, ARK Resource, Kumamoto, Japan) were evaluated at 21 days post parturition. The total antioxidant capacity can be used as an index of resistance to oxidative stress in animals. Here, the total antioxidant concentration (TAC) in milk was assessed using a TAC assay kit (Metallogenics, Chiba, Japan) during the lactation period. This kit uses ascorbic acid as an indicator of reduction power by reducing copper [31]. The unit of reducing power is expressed as equivalent to 1M of ascorbic acid [32].

Statistical analysis

All data are expressed as means ± SEM. Analysis of variance was used to test the overall effect of tempol. Unpaired comparisons using Student's *t*-test were used to determine the significance of differences between specific groups. Analyses were performed using StatView (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was considered significant.

Results

Changes in body weight and BP during pregnancy and lactation

In both the control and tempol-treated groups, mean body weight increased 1.3-fold by day 14 of pregnancy compared with the pre-pregnancy value (Fig. 2A). The two groups of rats showed similar changes in body weight throughout the lactation period, with no significant difference between the groups. In the tempol-treated group, SBP was consistently lower than in the control group, but this difference was only significant on day 7 of pregnancy ($P<0.05$). On day 14 of pregnancy, control females showed an approximately 10 mm Hg decrease in SBP compared with day 7; in the tempol-treated group, SBP was similar at both testing intervals (Fig. 2B). Furthermore, the SBP of the nursing rats in the tempol-treated group was significantly lower ($P<0.05$) on days 7 and 21 than in the control group. The DBP of the pregnant rats in the tempol-treated group was slightly, but consistently, lower than that in the control group (Fig. 2C). DBP was also significantly lower ($P<0.05$) on day 14 of nursing in the tempol-treated rats than in the control group.

Litter size on birth and sexing

The number of offspring did not differ significantly between the two groups of rats (Table 1). The sex ratio of the offspring was similar in both groups, with no significant difference between them.

Changes in the volume of milk produced and the total antioxidant concentration and 8-OHdG in the milk

There were no significant differences in milk volumes between the tempol and control groups throughout the experimental period (Fig. 3A). In both groups, the milk volumes increased by 1.5- to 2.0-fold at 14 and 21 days after parturition compared with at 7 days after parturition. Antioxidant activities in milk during the lactation period were measured (Fig. 3B). In ascorbate equivalents, the antioxidant activity at 7 days after parturition was significantly higher ($P<0.01$) in the tempol group (0.95 ± 0.09 mM) compared with the control group (0.38 ± 0.10 mM). Similarly, the TAC in the milk at 14 days after parturition was significantly greater in the tempol-treated rats than in the controls ($P<0.05$). Next, we evaluated the degree of oxidative stress by measuring the level of 8-OHdG in milk throughout the experimental period. There was no significant difference in the levels of 8-OHdG in the milk of the two groups (Fig. 3C).

Urinary levels of 8-OHdG and corticosterone in adult females

Analysis of urine collected over a 24h period showed that the level of 8-OHdG in the tempol-treated adult female rats was significantly lower than that in the control group ($P<0.01$; Fig. 4A). The level of corticosterone, which is closely associated with the degree of stress, was also measured in the 24h urine samples. The corticosterone level of the adult female rats in the tempol group (177.5 ± 34.7 ng/ml) was lower than that of the adult female rats in the control group (194.0 ± 15.5 ng/ml), but the difference was not significant (Fig. 4B).

Urinary levels of 8-OHdG and corticosterone in neonatal rats

In the neonatal rats, the urinary level of 8-OHdG tended to decrease in the tempol-treated group compared with the control group, but the difference was not statistically significant (Fig. 5A). However, the urinary level of corticosterone in the tempol-treated group was significantly lower than that in the control group ($P<0.01$; Fig. 5B).

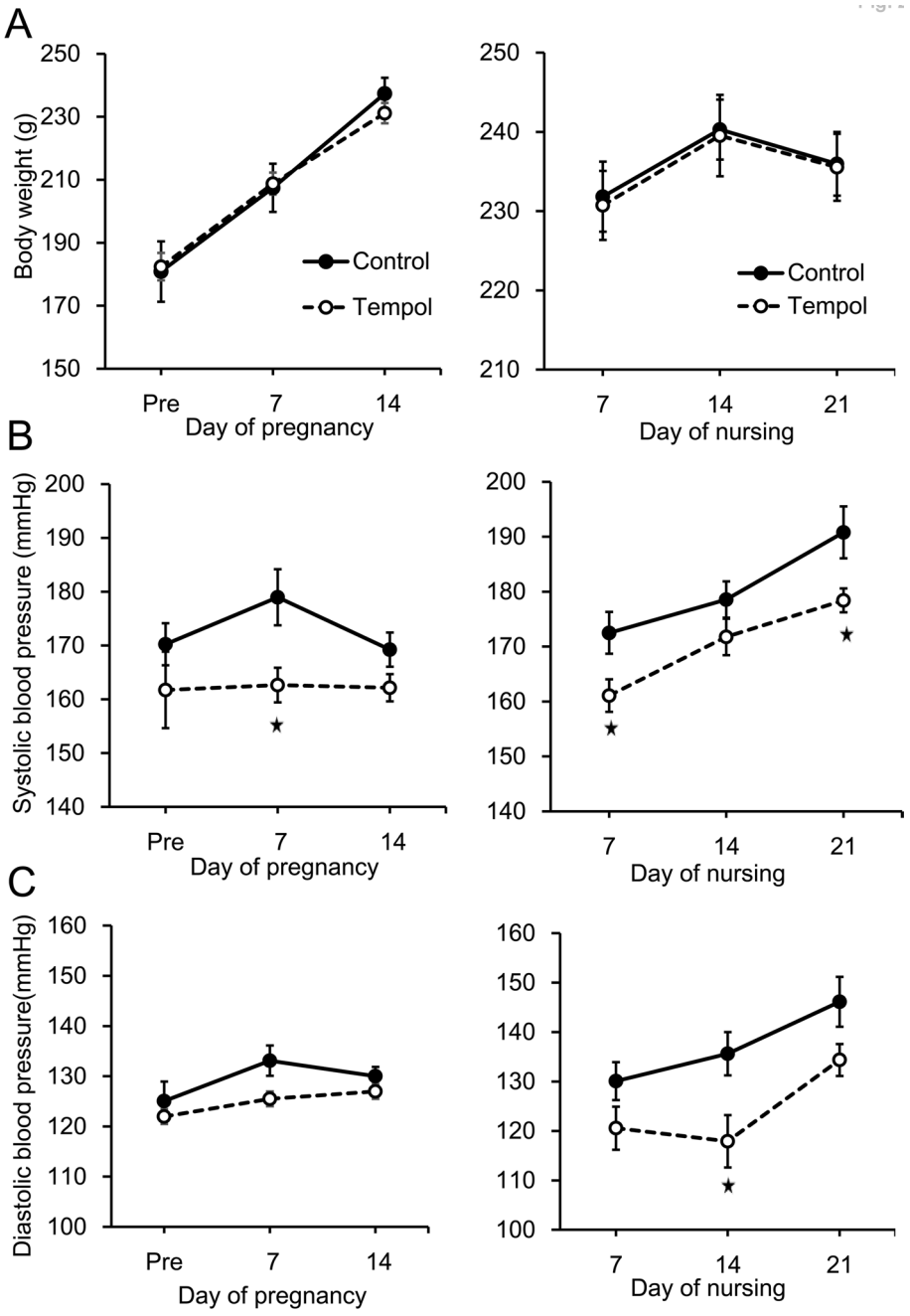

Fig. 2. Changes in body weight (A), systolic blood pressure (B), and diastolic blood pressure (C) in female rats during pregnancy and the lactation period. Rats were separated into 2 groups (8 animals in each): one group was given distilled water (control group), and the other was given tempol (antioxidant group). Values represent means ± SEM of 8 rats. Significant differences from the control group using Student's *t*-test. * $P<0.05$. Pre, non-pregnant rats.

Table 1. Effect of tempol on litter size

Group	Control	Tempol
No. of pregnant females	8	8
Average litter size	9.10 ± 0.70	9.80 ± 1.00
No. of male offspring	3.90 ± 0.60	4.30 ± 0.60
No. of female offspring	4.50 ± 0.70	4.90 ± 0.50

Values are presented as numbers or means ± SEM.

Plasma levels of CRF in adult females

As exposure to a variety of stressors markedly activates the sympathoadrenal and hypothalamic-pituitary-adrenocortical systems, we analyzed the plasma levels of CRF in the adult females. The analysis indicated that the plasma levels of CRF were below the detection limit (0.078 ng/ml) in both groups of rats (data not shown).

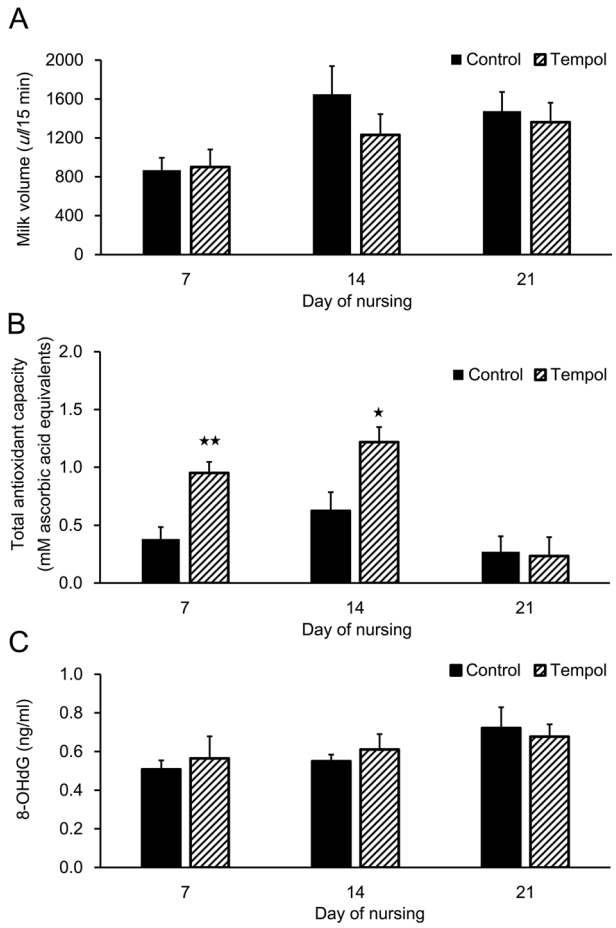

Fig. 3. Comparison of milk volume (A), total antioxidant capacity in milk (B), and 8-OHdG levels in milk (C) from female rats during the lactation period in the control and tempol-treated groups. Values represent means ± SEM of 8 rats. * P<0.05 (tempol versus control group).

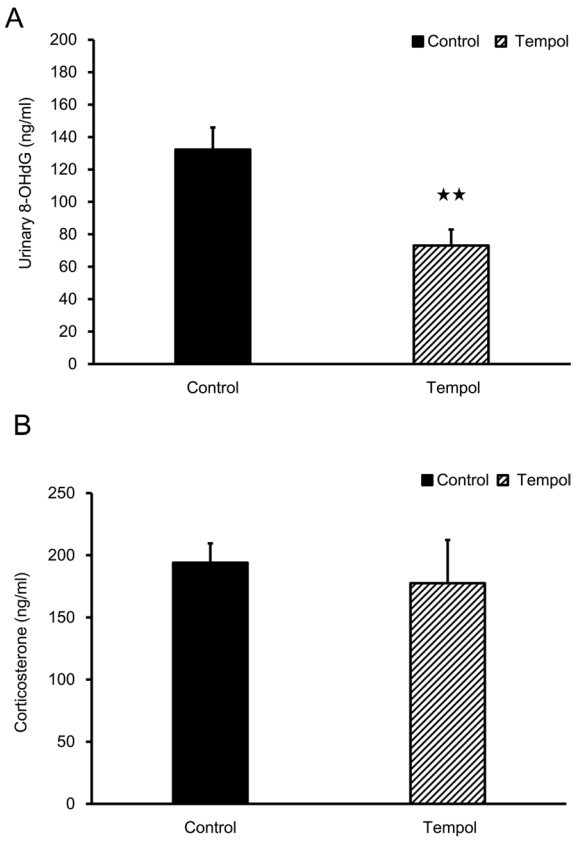

Fig. 4. Urinary 8-OHdG levels (A) and urinary corticosterone levels (B) in female rats after the nursing period. Metabolic cages were use to collect 24h urine. Values represent means ± SEM of 8 rats. ** P<0.01 (tempol versus control group).

Discussion

In this study, we investigated the effects of administration of tempol, an antioxidant, to SHRs during pregnancy and postpartum by measuring the changes in BP, litter size at birth, oxidative stress, and total antioxidant concentration in milk. We found that rats showed a decrease in BP, suggesting that maternal oxidative stress was reduced by administration of tempol during pregnancy. Treatment of pregnant females with tempol also reduced stress in neonates. Our findings indicate that antioxidant ingestion during pregnancy leads to a reduction in hypertension.

In the tempol-treated group, SBP and DBP were consistently lower than in the control group during pregnancy and in the post-natal nursing period. Tempol is a cell-permeable substance that eliminates ROS [24]. The hypotensive action of tempol has been suggested to be due to vasodilation, which is related to the inhibition of ROS production, sympathetic inhibition, and natriuresis [23]. A number of pathways can lead to an increase in ROS release; one of these is NADPH oxidase activity in vascular smooth muscle, which is enhanced by angiotensin II. In NADPH oxidase KO mice, vasopressor reactions for angiotensin II are reduced by half compared with wild-type mice, strongly suggesting that ROS is involved in vasopressor reactions for angiotensin II or angiopathy [33]. In humans, mutations in the NADPH oxidase gene are involved in the development of hypertension or cardiovascular disorders [34]. Our study demonstrated that continuous administration of tempol to pregnant SHRs reduced maternal oxidative stress.

We examined the potential harmful effects of tempol treatment during pregnancy by screening the number of live-born offspring and the sex ratio of the offspring; we also monitored the body weights of the pregnant rats. We confirmed that tempol treatment had no apparent deleterious effects on pregnancy. We also showed that the volume of milk and female body weights during nursing were similar in both groups. Although we did not measure pup weights in this study, a previous study on a mouse strain with fetal growth restriction showed that

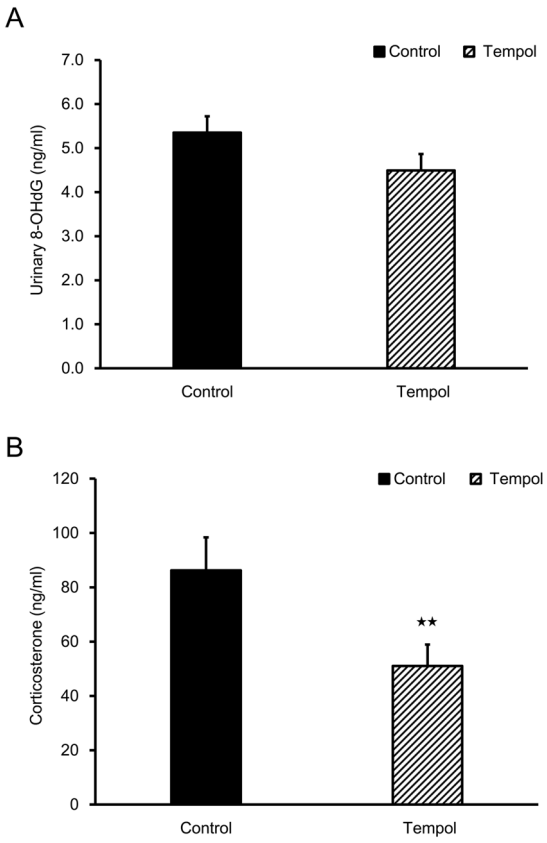

Fig. 5. Urinary 8-OHdG levels (A) and urinary corticosterone levels (B) in neonatal rats at 21 days after birth. Urine was collected from neonatal rats at 21 days post birth using the single sampling method (n=14–16 per group). Values represent means ± SEM. * $P<0.05$ (tempol versus control group).

tempol treatment resulted in an increase in pup growth [35]. Thus, antioxidant therapy during pregnancy might be able to at least partially rescue fetal growth deficiencies.

Our study also demonstrated that treatment of pregnant/lactating rats with tempol increased the total antioxidant concentration in milk. Furthermore, the total antioxidant concentration at 14 days post postpartum was slightly higher than at 7 and 21 days in both groups of rats. Previous studies reported that the antioxidant concentration in mature human breast milk was higher than that in colostrum [15, 36]. Milk contains many antioxidant substances, such as catalase, superoxide dismutase, ascorbic acid, and vitamin E [37]. Some of these antioxidant substances may be involved in ROS elimination in neonatal rats.

We also evaluated the level of 8-OHdG in milk. It has been suggested that an *in vivo* mechanism for synthesizing antioxidant substances from blood and condensing them into breast milk might be present in rats [38]. 8-OHdG is produced during the repair of intracellular nucleotide damage and is released into the extracellular area [24]. As milk is produced from plasma components and can be noninvasively collected, it can potentially be used for 8-OHdG analysis in addition to urine [39]. The level of 8-OHdG in the milk samples analyzed here did not show any marked difference between tempol-treated and control animals. On the other hand, urinary 8-OHdG is synthesized in tissues and organs under excessive oxidative stress [25, 40]; 8-OHdG is normally cleaved by repair enzymes and ultimately excreted into urine via the circulation. Therefore, to investigate whether the antioxidative action was involved in the inhibitory action of tempol on BP elevation, urinary 8-OHdG levels were measured in dams and neonatal rats. Post-nursing adult females had significantly lower 8-OHdG levels in the tempol group compared with the control group; urinary 8-OHdG levels in the neonatal rats tended to be low.

The urinary level of corticosterone in the neonatal rats was found to be significantly lower in the tempol-treated group than in the control group. Corticosterone is synthesized in response to stimulation of the adrenal cortex by adrenocorticotropic hormone (ACTH) and is a precursor for aldosterone [41]. Exposure to a variety of stressors markedly activates the sympathoadrenal and hypothalamic-pituitary-adrenocortical systems in animals [42]. Stressful stimuli activate the hypothalamo-pituitary-adrenocortical axis and release glucocorticoids, such as corticosterone [43]. Also, maternal insults, such as malnutrition or stress (physiological and psychological), can permanently alter tissue structure and function and can lead to cardiovascular dysfunction in the fetus [44]. Roghair *et al* [45] showed that maternal tempol treatment via drinking water reduced glucocorticoid-mediated cardiovascular responses in mice. In view of the above, the purpose of our CRF measurements was to investigate mechanisms in relation to corticosterone in the hypothalamic-pituitary adrenal axis. However, the CRF levels were below the level of detection in both groups. Fukuda *et al* [46] showed that SHRs have an abnormal response to CRF in the pituitary-adrenocortical axis and that this abnormal response may be attributable to desensitization of the pituitary to CRF. Further studies will be necessary to fully elucidate the mechanisms.

In conclusion, our analysis showed that continuous administration of tempol to pregnant SHRs inhibited the pregnancy-related increases in BP. Furthermore, maternal oxidative stress was lowered, as was that in the neonatal rats. In future studies, the effects of antioxidant treatment on the growth of neonatal rats and on their development under different environments will be investigated.

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Conflict of Interest

The authors declare that they have no competing interests.

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