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Effect of Low Maternal Aldosterone on Offspring Cardiovascular Development

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EFFECT OF LOW MATERNAL ALDOSTERONE ON OFFSPRING CARDIOVASCULAR DEVELOPMENT

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Abstract

Effects of low maternal aldosterone on offspring cardiovascular development.

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Maternal conditions are well known to affect fetal cardiovascular development. This study was designed to investigate in spontaneously hypertensive rats (SHR), the effects of maternal aldosterone suppression on the development of hypertension in the offspring. A sham procedure or a right adrenalectomy combined with cryodestruction of the zona glomerulosa of the left adrenal gland was performed on SHR females. The rats were mated and male offspring were implanted at 11-13 weeks of age with a PA-C40 remote monitoring device placed into the femoral artery to record cardiovascular parameters for 4 weeks. Systolic (mmHg), diastolic (mmHg), and mean blood pressure (mmHg), heart rate (beats per minute), and activity data was measured. Data was sorted by activity level, and parameters were compared to evaluate same age data points between freeze and sham groups. Continuous scatterplot with regression lines compared activity level versus mean pressure, pulse pressure, and heart rate for each of the four weeks of data collection. The slopes and elevations of the regression lines from the sham and frozen groups were compared using a two-way ANOVA. Reductions in maternal aldosterone concentrations in the adrenal frozen group led to changes in adult offspring hemodynamic profiles. The adult offspring of the adrenal frozen group were found to have an attenuated increase in mean pressure with activity during weeks 2 and 3, and an overall lower mean pressure during week 4. It appears that a difference in sympathetic activation was likely responsible for the differences between groups, and this fetal programming effect has not been observed with other maternal manipulations.

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Introduction

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States. The Center for Disease Control and Prevention (CDC) reports that in the U.S. approximately 26% of the annual mortality rate is caused by coronary heart disease-related illness (1). In 2010, the United States spent approximately $316.4 billion dollars on cardiovascular disease related-care including the cost of health care services, medications, and loss of productivity (2). The proliferation of obesity in children and adolescents is also predicted to increase the cost of health care in the upcoming decades due to the rising number of adults that will require heart disease and type 2 diabetes related care (3). Hypertension is a major underlying determinant of cardiovascular disease, affecting approximately one in three adults in the U.S. (2). National Vital Statistics Survey (NVSS) reports that hypertension contributes to one out of every seven deaths in the United States each year (1). Hypertension additionally contributes to nearly half of all deaths related to cardiovascular disease (1).

Guyton et al. (1964) suggested a potential link between a reduction in renal sodium excretion and the pathogenesis of hypertension. The study found that the kidneys play an important role in the origin and maintenance of hypertension (4). Thornburg and colleagues found that a family history of hypertension and obesity are both risk factors commonly associated with the development of hypertension (5). The role of family history on hypertension and obesity in the renal handling of sodium was also evaluated. Both obese and lean adolescent children of hypertensive parents had increased proximal tubular sodium reabsorption in the nephron, which increased the likelihood of hypertension development (6). Woods and colleagues additionally used a rat model to demonstrate that the renin-angiotensin-aldosterone

system (RAAS), involved in sodium reabsorption through the action of aldosterone, can contribute to the development of adult hypertension (7). These studies support the theory that hypertension is caused by defects in sodium and fluid balance.

While people have the ability to decrease their risk of developing coronary heart disease through diet, exercise, and maintaining a healthy lifestyle, epidemiological evidence examining the association between birth weight and hypertension has demonstrated that slow rates of prenatal growth can significantly predict the likelihood of cardiovascular disease development (8). Furthermore, the correlation between blood pressure and birth weight appears to increase as a person ages (9). Fetal undergrowth may occur as a result of placental inadequacies (8). In addition, the nutritional and hormonal environment of the fetus is correlated with low birth weight and the subsequent development of hypertension and cardiovascular disease in adulthood (10). Events during fetal development have been shown to have long lasting impacts on tissue structure and function (9, 11, 12, 13).

Maternal conditions involving alterations of the RAAS or other adrenal hormones affect fetal development and subsequent offspring cardiovascular disease risk. Several studies suggest that the adrenal cortex hormones can contribute to renal defects of the maternal kidneys and produce hypertension in their offspring through the action of the RAAS (7, 11, 14). Low sodium diets in maternal rats have been shown to cause elevated aldosterone levels in the maternal and fetal blood, intrauterine growth restriction, and an increase blood pressure in the offspring (15, 16). Sodium depletion in maternal rats results in offspring that have a greater salt appetite, an effect shown to be dependent on the resultant elevation in the RAAS (17). Fetal synthesis of aldosterone is not present prior to day 18 of gestation, and the source of fetal aldosterone for most of the gestational period is from the maternal blood (18). Aldosterone crosses the maternal

feto-placental barrier, and therefore can affect fetal development (18). Through hormones including aldosterone, the kidney and adrenal gland are able to regulate gene expression, growth, and maturation of the fetus (11, 19).

Since it is established that intrauterine conditions can alter fetal development, and aldosterone appears to play a role in the development of hypertension, the current study will investigate the effects of maternal aldosterone suppression on the development of hypertension in spontaneously hypertensive rat (SHR) male offspring. There have been no previous studies that have investigated the effects of reduced maternal aldosterone on the development of hypertension in offspring. The current study, which uses maternal SHR subjected to a unilateral adrenalectomy and contralateral zona glomerulosa cryodestruction, is unique. The adrenal freezing will suppress only aldosterone because its synthesis and release is exclusive to the zona glomerulosa (20). However, this model allows aldosterone to respond to physiological stimulators/inhibitors, while levels remain at approximately 30% of normal (21). Other prenatal programming or intrauterine growth restriction (IUGR) studies aimed at investigating the effects of both the RAAS and aldosterone during fetal development have not used a surgical strategy that reduces the release of aldosterone secretion without disturbing other hormones synthesized in the adrenal cortex. A reduction in maternal aldosterone is likely to result in a lowered blood volume during pregnancy, which is predicted to elevate blood pressure in the adult offspring of the adrenal frozen rats as a result of intrauterine growth restriction from the compromised nutrient and hormonal environment in utero.

Chapter II

Literature Review

Hypertension

High blood pressure, otherwise known as hypertension, is the principal factor contributing to the morbidity and mortality associated with coronary heart disease, stroke, and kidney failure (22, 23). Arterial hypertension is characterized by having a level of blood pressure at 140/90 mmHg or higher, and is hypothesized to be associated with both genetic and environmental factors (23, 24). The correlation between blood pressure and hypertension is more related to the systolic measurement than the diastolic pressure measurement (22). General characteristics of essential hypertension, which without a defined underlying cause, include: increased vascular stiffness, increased vascular resistance, and increased vascular responsiveness to stimuli (23). Blood pressure is determined by both taking the product of cardiac output and peripheral vascular resistance, which regulates arteriolar blood flow into the capillaries (25). Therefore, the pressure is determined by the amount of blood being pumped from the heart into the circulatory bed (cardiac output), and the vascular tone or level of constriction applied on the arteries (systemic vascular resistance) (22). Total peripheral resistance, or the overall vascular tone, may be elevated due to an increased release of peptides such as angiotensin, endothelins, or increased α-adrenoceptor stimulation (23).

The cardiac output is determined by the stroke volume and the heart rate (22). If the heart rate and/or the stroke volume increase, then cardiac output will increase as well. The increase in cardiac output associated with hypertension arises from an increase in fluid volume, or from an increase in contractility correlated with neural stimulation of the heart (22).

The autonomic nervous system is also important for the control of blood pressure. The sympathetic branch increases heart rate, stroke volume, and vascular resistance to raise blood pressure. The parasympathetic branch tends to decrease heart rate and lower blood pressure. In patients with hypertension there is often an increased peripheral sensitivity to the sympathetic neurotransmitter norepinephrine, and an increased responsiveness to physiological stressors (22, 23).

The activation of the renin-angiotensin-aldosterone system (RAAS) also increases angiotensin II in blood to cause vasoconstriction, which results in a rise in vascular resistance and an elevated blood pressure. Angiotensin II stimulates the release of aldosterone from the adrenal cortex, which acts on the kidneys to cause salt/water retention, an increase in blood volume, and an increase in cardiac output (26). Blood pressure at the kidney controls for fluid retention independent of hormonal influence, and is the dominant mechanism for long-term blood pressure control. Therefore, when blood pressure rises, body fluid volume drops in order to reduce blood pressure at the level of the kidneys to normal (27). The increased sodium excretion in response to a rise in blood pressure is known as pressure natriuresis (23).

Kidney Function

The kidney excretes metabolic waste products and maintains plasma osmolarity (28). The kidney contains functional units called nephrons that filter plasma, reabsorb water and nutrients, and secrete wastes. The nephron contains a tubule that extends from structure in the kidney called the renal corpuscle, consisting of the Bowmann's capsule and glomerulus, which filters fluid (filtrate) out of the blood and into the tubule (28). The filtrate is altered by selective

reabsorption, and water and nutrients not reabsorbed back into the kidney tubules are excreted in the urine (29). Regulation of reabsorption is under the control of several hormones, including ADH (antidiuretic hormone), atrial natriuretic peptide (ANP), ANG II, and aldosterone (30). ADH promotes the reabsorption of water through the insertion of channels which allow for the movement of water through the cell membranes of the collecting duct, back into the capillaries (28). Aldosterone increases potassium secretion and sodium reabsorption (20, 22).

Because the regulation of sodium and water are necessary to maintain fluid balance, the kidneys are important for long term control of blood pressure (30). There are two mechanisms that regulate sodium handling in the kidney. One mechanism regulates fluid volume by increasing or decreasing the excretion of sodium and water which changes the renal perfusion pressure. This mechanism is also called pressure natriuresis (23, 29). The second mechanism utilizes the renin-angiotensin-aldosterone system to directly control peripheral resistance and the renal reabsorption of sodium and water through the action of aldosterone (29). At the distal tubule, aldosterone acts to increase sodium reabsorption, and is involved in fluid retention and blood pressure regulation. Excess secretion of aldosterone enhances sodium reabsorption, and can lead to the development of hypertension by increasing blood volume and cardiac output (31).

Renin-Angiotensin-Aldosterone System (RAAS)

The renin-angiotensin-aldosterone system (RAAS) plays an important role in salt and water homeostasis (12, 20, 32, 33). The RAAS is an important system in the development of hypertension. Based on the commonly understood RAAS cascade, depressed levels of renin secretion will decrease angiotensin I (ANG I) and II production, reduce aldosterone secretion,

and decrease blood pressure. Renin belongs to a family of aspartyl proteases that is highly specific for angiotensinogen (33). Renin secretion is influenced by the pressure of the renal artery via the vascular baroreceptor (12). Over and underproduction of the enzyme renin results in disturbances in plasma osmolarity and volume, and alterations in blood pressure (33). The majority of renin synthesis occurs in the kidney; however, several other organs including the brain, pituitary, adrenal glands, and arterial smooth muscle also contribute small amounts (12).

Angiotensinogen is converted to angiotensin I (ANG I) by renin, and angiotensin I is converted to angiotensin II (ANG II) by angiotensin converting enzyme (ACE), located on the pulmonary vascular endothelium (32). Angiotensinogen is a glycoprotein stored and released by the liver (34). Angiotensin II stimulates the release of aldosterone from the adrenal cortex, which regulates sodium reabsorption in the kidney. The release of angiotensinogen is also stimulated by ANG II (33).

ANG II can stimulate plasma aldosterone secretion through the RAAS cascade. Aldosterone acts to increase sodium reabsorption and potassium excretion in the distal tubule of the kidney. Sodium reabsorption is known to increase blood volume and blood pressure (32). ANG II secretion would be expected to be accompanied by increases in plasma aldosterone. Plasma aldosterone concentrations were compared in rats administered ANG II under salt loading and normal conditions (32). Plasma aldosterone concentrations did not change, regardless of ANG II administration. Therefore, small increases of ANG II do not necessarily lead to an increase in plasma aldosterone levels, indicating a more complex regulation process for aldosterone release (32).

Sodium-intake variations are likely to influence plasma aldosterone concentrations. Wambach et al. (1982) observed the aldosterone excretion of 100 hypertensive patients fed a

high-sodium diet (35). The first group of patients (n=64) had suppressed aldosterone excretion similar to the normotensive control patients. The second group (n=36) had aldosterone levels above the control patients and serum potassium levels were lower than the first group of patients despite forced sodium loading. However; the second group responded better to spironolactone (aldosterone receptor antagonist) treatment to reduce blood pressure, and ANG II infusion was found to elevate plasma aldosterone level in both groups. Sodium loading may cause fluctuations in aldosterone concentrations which can resemble a normotensive patient, or a patient with hypertension (35).

Adrenal Cortex

The adrenal cortex consists of the outer portion of the adrenal gland. The cortex secretes hormones that potentiate certain chemicals in the blood, affect the rate of metabolism, and can cause changes in growth. The adrenal cortex secretes glucocorticoid and mineralocorticoid steroid hormones directly into the bloodstream (36). The cortex contains three distinct layers which release various steroid hormones: the innermost zona reticularis, the middle zona fasciculata, and the outer zona glomerulosa. The zona reticularis and fasciculata produce androgens and glucocorticoids, respectively. The zona glomerulosa secretes aldosterone (36).

Both corticosterone and aldosterone have been linked with hypertension. Cortiocosterone is secreted in response to stress, stimulated by ACTH from the anterior pituitary (20, 36, 37). Increases in corticosterone have been associated with increased cardiovascular risks including the development of hypertension (33). Aldosterone acts to increase the retention of sodium and water in the kidneys, therefore it also plays an important role in the development of hypertension (20). Sodium depletion or potassium elevation results in stimulation of aldosterone

secretion. The zona glomerulosa has been found to hypertrophy following sodium reductions and/or elevations in potassium, and the cells become hypersensitive to ANG II under these conditions (38). Studies have found that aldosterone reduction may inhibit or delay the development of hypertension by decreasing blood volume and blood pressure (14, 20). Aldosterone exerts its activity by binding to the mineralocorticoid receptor (MR). Corticosteroid is able to bind the same receptor; however, 11 beta-HSD type 2, co-localized with the MR receptor, inactivates glucocorticoids, thus preventing inappropriate activation (37).

Hypertension and Prenatal Programming

Hypertension, insulin resistance, type 2 diabetes, and other factors linked to cardiovascular disease are commonly associated with lifestyle choices in adulthood (13). However, an increasing body of epidemiological evidence suggests that the nutritional and hormonal environment of the fetus is correlated with low birth weight and the subsequent development of hypertension and cardiovascular disease in adulthood (10). Events during fetal development have been shown to have long lasting impacts on tissue structure and function (9, 11, 12, 13). The association between the development of hypertension, type 2 diabetes, and cardiovascular disease with reduced fetal growth has been found to be independent of life style-associated risk factors including smoking, obesity, alcohol consumption, and socioeconomic status (39).

Reduced fetal growth has been associated with an increased risk for hypertension (8) Barker showed that the mean systolic and diastolic blood pressure of men and women was elevated in the cohort with low placental weights. Low placental weight was found to be associated with low birth weight (8). Evidence of birth weight on the development of

hypertension is further supported using data from individuals with birth weights within normal ranges (40, 41). These studies found that individuals with normal birth weights were less likely to develop hypertension independent of lifestyle choices. Gluckman et al. (2004) used identical twin birth weight data to investigate the development of hypertension in adulthood (42). The twin with the lowest birth weight was found to have the highest blood pressure as an adult. The growing body of research showing the significance of prenatal programming on the development of hypertension does not suggest that birth weight is the direct cause of disease, rather that fetal programming processes are a major contributing factor to the prevalence and development of disease (42, 43).

Maternal Protein Deficiency and Prenatal Programming

Maternal protein deficiency appears to be important in fetal growth restriction and the development of hypertension in the offspring (11, 44, 45). Under moderate protein restriction, an increase in offspring blood pressure by 20-30 mmHg was found (11). The hypertensive effect was mediated by exposure to an excess of maternal glucocorticoids as a result of an 11-beta-HSD 2 deficiency in the placenta. 11-beta HSD2 converts corticosterone to an inactive form, 11-dehydrocorticosterone, and protects the fetus from being exposed to excess corticosterone (11). Benediktsson et al. (1993) suggested that corticosterone excess during fetal development is associated with low offspring birth weight. The low birth weight described is closely associated with the development of cardiovascular disease in adulthood (43, 46).

McMullen et al. (2004) also investigated the offspring of low-protein fed mothers. Pregnant WKY rats were fed a control diet or a low-protein diet during gestation (45). The

nephron number in kidneys in 4 week old offspring was significantly reduced under maternal protein restriction. These offspring had greater systolic blood pressure than controls. The offspring of low-protein fed mothers also had low renal expression of the angiotensin II type 2 receptor, and elevated blood pressures. The type 2 receptor tends to have the opposite effect on sodium retention as the more prevalent angiotensin II type 1 receptor. The authors suggest that the lower expression of this receptor may be important for renal development impairment and hypertension exhibited in the offspring of mothers fed a low-protein diet during pregnancy (45).

The RAAS and Fetal Development

Lumbers (1995) suggested that the renin-angiotensin-aldosterone (RAAS) system varies significantly during intra-uterine and extra-uterine life. During pregnancy, the fetus is affected by the mother's RAAS. The maternal RAAS is activated when there are severe losses in sodium and fluid volume. The fetal kidneys function to deliver fluid to the amniotic cavity to form amniotic fluid. The amniotic fluid environment is important to allow for chest cavity movement, the free movement of limbs, and prevents the uterine wall from wrapping tightly around the fetus (14). Lumbers also found that ATII levels in fetal sheep are similar to maternal levels and the fetal RAAS is important for blood pressure maintenance. The study also found that secretion of aldosterone from the fetal adrenal is only stimulated with high doses of AII (14).

Reductions in maternal sodium intake during pregnancy have been shown to affect the maternal and fetal RAAS. Several studies have investigated intrauterine growth restriction (IUGR) and the development of hypertension in rats subjected to an altered maternal sodium diet (16, 47, 48, 49). Battista et al. (2002) examined blood pressure, RAAS activity, renal function

and heart and kidney histology in 12 week old offspring born from pregnant rats fed a low sodium diet, resulting in IUGR offspring (16). Systolic blood pressure was elevated, urinary sodium excretion was decreased, and aldosterone levels were not found to be correlated with renin activity in the IUGR male and female adult rats. Based on these findings, the authors suggested that IUGR-induced offspring of pregnant rats fed a low-sodium diet are prone to alterations in the RAAS, elevations in blood pressure, and impairments in renal activity in adulthood (16).

Increases in sodium intake during pregnancy may also affect the RAAS of the offspring. Ramos et al. (2000) looked at the RAAS response to a salt loading in maternal WKY rats. Female WKY were either fed a high-sodium (8%), or normal-sodium (1.3%) diet from 8 weeks of age until the birth of their first litter, and offspring were fed a normal-sodium diet until 12 weeks of age (47). At 12 weeks of age, the offspring were fed either a high-sodium or low-sodium (0.16%) diet for one week to evaluate the RAAS response and sodium excretion. Blood pressure was significantly higher in the high-sodium mothers than normal-sodium mothers, but no blood pressure differences in the offspring were observed. Male offspring were found to have increased blood pressure after one week of high-sodium feeding, but the change was found to be independent of maternal diet. In female offspring of maternal dams fed a high-salt diet, sodium excretion was reduced in response to salt overload in the high-salt female offspring, but not in the normal-salt females. Therefore, the authors concluded that RAAS responsiveness to maternally-fed high sodium diets are gender specific in the adult offspring. (47).

Aldosterone and the Fetal Environment

The maternal circulation is the source of aldosterone for the fetus for much of gestation. The development of the rat adrenal gland in utero was studied using the expression of aldosterone synthase cytochrome P450 (P450aldo), a functional marker for the expression of mineralocorticoid-synthesizing zona glomerulosa cells (18). P450aldo was not detected until gestational day 20, only 2 days prior to birth. However, histological analysis found that zona glomerulosa cells were present at day 18 (18). The establishment of rat adrenal gland maturation and cortex zonation was analyzed in utero using markers for the adrenocortical zones (50). Analysis of the adrenal cell markers showed that functional zonation of the cortex did not develop until around the time of birth, although cells that synthesize the DNA for adrenal cortex hormones were found throughout the gland before zonation was established. These findings suggest that the fetal synthesis of aldosterone is at least not present prior to day 18 of gestation, and the source of fetal aldosterone is from the maternal circulation.

Decreased maternal sodium intake can increase aldosterone levels in the fetus. Bibeau et al. (2010) evaluated the expression of proteins and enzymes in the adrenal glands of IUGR rat fetuses. IUGR fetuses responding to decreased maternal sodium diet were found to have increased aldosterone concentrations, and were associated with higher adrenal levels of angiotensin II receptor type 1 and cytochrome P450 aldosterone synthase. It is likely that the alterations observed in these IUGR fetuses would affect the development of hypertension in adulthood (15).

Maternal aldosterone deficiency during pregnancy has been found to affect offspring development in mice (51). During pregnancy, high aldosterone concentrations lead to an expanded plasma volume, which is important for fetal nutrition. Todkar and colleagues used

aldosterone synthase deficient pregnant mice to observe the effects of low aldosterone on the mother and fetus. The study found that maternal systolic blood pressure was low prior to pregnancy and was further reduced during pregnancy. The aldosterone synthase deficient mice were found to have smaller litters, and a higher number of necrotic placentas with high lymphocytic infiltrations at gestational day 18, which indicated a loss of fetuses. A high-sodium diet administered before and during pregnancy increased systolic blood pressure, improved differences in fetal and placental weights, and improved litter size. The result suggested that aldosterone deficiency has strong adverse effects on the fetal environment (51). The study did not examine the effects of reduced aldosterone on offspring development of hypertension.

Finally, the effect of maternal aldosterone on trophoblast formation and growth and feto-placental function was investigated (52). Gennari-Moser et al. (2011) hypothesized that low aldosterone and high cortisol associated with pregnancy-induced hypertension may have an effect on placental growth and function. Functional deficiency in aldosterone was created in pregnant mice by spironolactone treatment, a mineralocorticoid receptor antagonist. Spironolactone treatment led to reduced fetal umbilical blood flow. These findings suggested that the activation of the mineralocorticoid receptors by maternal aldosterone is required for normal feto-placental function (52).

The Spontaneously Hypertensive Rat (SHR)

Okamoto et al. (1964) developed the spontaneously hypertensive rat (SHR) strain in order to provide an animal model of hypertension that requires no surgical or pharmaceutical treatment. The investigation showed that the SHR underwent pathological changes similar to

those observed in human hypertension, including nephrosclerosis, vascular damage, and cardiomegaly (53). Another study found that rats utilize similar mechanisms for sodium regulation as their human counterparts (54). The portion of the adrenal cortex responsible for the production and synthesis of aldosterone has been found to be larger in the SHR strain than that of the normotensive Wistar rat (WKY) strain (55). The differences observed in the size of the zona glomerulosa (outer adrenal cortex) suggests a link between the action of the RAAS and the development of hypertension (7, 11, 55, 56).

The intrauterine environment is altered in the SHR (57). Fetal and placental growth and amniotic fluid composition in SHR and WKY (control) rats was investigated to determine the role of the intrauterine environment on the development of hypertension in SHR. Fetal and placental weights of SHR were found to be significantly lower than the WKY controls. Amniotic fluid volume, sodium concentration, and potassium concentration were significantly decreased in SHR at gestational day 15 compared to the control group. However, amniotic fluid sodium concentration was not different in SHR and WKY rats at gestational age 20. It still remains unclear whether the changes in the placenta and amniotic fluid are of fetal or maternal origin. It is also unclear how the findings link fetal development to adult blood pressure (57).

The renin-angiotensin-aldosterone system (RAAS) is the primary means to regulate renal sodium. An excessive retention of sodium has been found to contribute to hypertension development (33). Mortiz et al. (2000) found that changes in the kidney and handling of renal sodium are often sex specific, and these differences can result in observed sex differences in disease outcomes and severity (12). By selecting only male offspring in this study, any influence that sex has on the development of hypertension can be eliminated.

Models of Reduced Aldosterone

Reductions in aldosterone, or aldosterone effects are produced by pharmacological, genetic, or surgical means. Pharmaceutical treatments to reduce aldosterone effects include MR blockers such as spironolactone or eplerenone, or synthesis inhibitors. Ménard and colleagues investigated the effectiveness of aldosterone-synthase inhibitors using 8 week old male SHR (58). The SHR were split into two groups; one fed a low sodium-high potassium diet to induce high urinary aldosterone, and the other fed a high sodium-normal potassium diet to induce low urinary aldosterone. FAD 286 A (10 and 30 mg/kg) aldosterone-synthase inhibitor was found to decrease plasma aldosterone by 53 and 87% on the low sodium diet, and 50 and 87% on the high sodium diet. Therefore, aldosterone-synthase inhibition appeared effective in reducing aldosterone in the SHR.

Aldosterone receptor blockers are used to decrease the effects of aldosterone on the kidney. Ortiz and colleagues evaluated the effects of aldosterone receptor blockade with eplerenone on renal sodium excretion, blood pressure, plasma aldosterone, and renal ANG II in rats (59). Eplerenone was found to increase sodium excretion within 24 hours in both normotensive (control) and SHR rats but, alternate mechanisms for sodium reabsorption were activated within 5 days to reestablish sodium balance. Eplerenone was found to increase intra-adrenal ANG II and aldosterone content. The elevated intrarenal ANG II contributed to sodium reabsorption, and partially explains the ineffectiveness of the aldosterone-receptor blockade in lowering systolic blood pressure in ANG II-infused hypertension models (59).

Surgical means to reduce aldosterone include complete adrenalectomy (often with hormone replacement) and adrenal enucleation. Adrenalectomized pups without steroid replacement exhibited impaired urinary-concentrating ability, a smaller renal medulla, and a lower medullary

interstitial osmolality (60). Enucleation of the adrenal involves removing the medulla from the gland, and allows the cortex to regenerate, eventually producing glucocorticoids and mineralocorticoids (61).

All models of reduced aldosterone are at least temporarily effective in reducing aldostone levels or aldosterone effects. However, none of this model allows aldosterone responses to physiological stimulators, while keeping aldosterone levels below normal. The adrenalectomy/adrenal freezing procedure developed in this laboratory reduces aldosterone levels by approximately 70%, while still allowing responses to physiological stimulators such as low sodium, angiotensin II, or stress (21).

Current Study

The current study was designed to investigate the effects of maternal aldosterone suppression on the development of hypertension in male offspring. A unique surgical means involving unilateral adrenalectomy and contralateral adrenal freezing was employed to reduce aldosterone in the maternal rats. Since reduced aldosterone is likely to result in a lowered blood volume during pregnancy, much like maternal sodium restriction, a higher blood pressure is predicted in the adult offspring of the adrenal frozen rats.

Chapter III

Materials and Methods

From the breeding colony, 8-10 week old SHR females were randomly divided into two groups, sham or adrenal frozen. A total of 18 maternal SHR from sham (n=10) and adrenal frozen (n=8) groups were used from the breeding colony at Minnesota State University, Mankato. All procedures were approved by the IACUC committee (Animal care number: 04-04). The SHR were allowed free access to standard rat chow (Lab Diet 5001; Purina Mills, Brentwood, MO) and tap water. The rats were housed in a constant temperature environment (20-22˚C) with a 12-hour light-dark cycle. Female SHR were paired with a male SHR for mating, and allowed to give birth. After the sham/freeze surgical procedure, the SHR females were reintroduced to a male for a second mating. Offspring of the second mating were studied beginning at 11-13 weeks of age.

Breeding

The SHR females underwent an initial mating, pregnancy, and parturition prior to the adrenal sham or freeze procedure. SHR often cannibalize pups from their first litter and offspring mortality during the first month of life was found to be higher in spontaneously hypertensive rat strains (50%) than in the WKY control (24%) strain (62). The pups from the first litter were removed and euthanized three to four days post birth. The maternal rat was then allowed to recover for 7 to 10 days before undergoing the adrenal freeze or sham surgery.

Adrenal Freeze/ Sham Surgical Procedures

The maternal SHR underwent either a sham or freeze surgical procedure 7 to 10 days after removal of the first pups. Half of the rats were subjected to the unilateral adrenalectomy/adrenal freeze surgery to reduce the production of aldosterone, and half underwent the sham surgery. The surgical procedures were previously developed in the laboratory of Dr. Penny Knoblich at Minnesota State University, Mankato, and have been shown to reduce unstressed aldosterone levels to 30% of normal in pregnant females that were adrenalectomized/adrenal frozen prior to mating and euthanized on day 20 of gestation (63). The removal of one gland provided optimal surgical reductions in aldosterone without significantly influencing corticosterone levels because the remaining gland hypertrophies to compensate (64). Each maternal rat was weighed in a plastic rectangular anesthetic box to the nearest gram on a triple beam balance. The following information was recorded in a laboratory notebook: rat, date of birth, the surgery date, sham/freeze, sex of the rat, and rat identification. Each rat was given a unique identification prior to the first introduction with a male. All identifications began with TM, which was followed by a letter designated in alphabetical order based on the rat's order of set up. The laboratory notebook was also used to note anything that may have occurred during the course of, or as a result of the surgery performed.

The rat was anesthetized with 3% isoflurane in oxygen (1 liter/minute flow), pumped into the plastic anesthetic box into which the rat was placed. Once the rat showed no response to rotations of the box, the rat was removed from the box and a face mask was taped to the rat's nose to supply the anesthetic/oxygen mixture. The oxygen and isoflurane was then readjusted: the oxygen was set to 0.5 L/min, and the isoflurane to 2.5%. Anesthetic levels were adjusted as needed to keep the rat at the proper depth of anesthesia.

Once the face mask was properly secured, the incision area, which includes the dorsal and lateral sides of the rat's back over the caudal edges of the ribs, was shaved using an electric shaver. The shaved hair was removed with a vacuum to eliminate hair from contaminating the surgical site. The rats were then placed on a standard heating pad that was adjusted as needed to maintain rectal temperature at 36-37˚C, measured using a thermometer with a rectal probe (Digital Thermometer; VWR Scientific). The incision area was then scrubbed three times with a surgical disinfectant (Chlorhexidine Scrub; Priority Care) diluted in water, and was followed each time by a distilled water rinse. Cleansing started from the site of the incision and moved outward. Sterile procedure was used by the surgeon. The surgeon scrubbed three times with the disinfectant soap using a sterile scrub brush. The surgeon then used a sterilized disposable cloth to dry and sterile disposable gloves were put on both hands. The rat was covered in a sterile disposable drape with the appropriate sized hole cut over the incision site. All tools used during the procedures were sterilized by autoclave prior to surgery.

Cryodestruction of the Left Adrenal Zona Glomerulosa

The adrenal freeze procedure consisted of an adrenalectomy on the right side of the rat combined with the cryo-destruction of the outermost left adrenal gland cortex. An approximately 2.5cm incision was made through the skin on the left side 3-5mm caudal to the ribcage using a sterile scalpel (#10). Using blunt dissection with a mosquito forceps, the layers of muscle located beneath the skin were separated until the internal organs were visible. Once the internal organs were exposed, a spring type tissue retractor was inserted to hold the skin and muscle tissue open to facilitate isolation of the adrenal gland. The left adrenal gland was located

using sterilized cotton swabs to manipulate the viscera. The adrenal gland was then retracted by using a metal hook that was placed around the adrenal vein and ligaments. Fat was cleaned off the gland, and a thin metal spatula (5mm wide) was dipped into a solution of liquid nitrogen and pressed gently against the gland to freeze the outer cortex. Each area of the surface of the gland was frozen three times, overlapping the sections covered by one application of the spatula to ensure complete cryodestruction. After the left adrenal gland was frozen, the gland was returned to its original position inside the body. The adrenal vein was inspected for bleeding before the incision was closed. The muscle was closed using a sterile absorbable polyglycolic acid suture (4-0; 3/8 circle cutting needle; Syneture). The skin was then closed with sterile monofilament nylon suture (4-0; 3/8 circle cutting needle; Henry Shein).

Right Side Adrenalectomy

After completion of the left adrenal freezing, the rat was repositioned onto its left side with the right side up. The right incision was made identically to the left, 3-5mm caudal to ribcage. Once the internal organs were again exposed, the adrenal gland was isolated using gentle dissection with sterile cotton swabs. The adrenal vein was then clamped off using a mosquito forceps. The adrenal artery, vein, and ligament were cut off distal to the forceps after time was allowed for venous blood to clot, and the gland was removed. The adrenal vein was then inspected for bleeding prior to incision closure. The right side incision was closed in the same manner as the left incision. All rats were given buprenorphine (0.3mg/kg, subcutaneously) post-surgery to reduce pain.

Sham Surgery

A sham surgical procedure was performed on the control rats to account for the effects of surgery alone. SHR were surgically opened using the same procedure as explained in the freeze group, however this group was sutured closed without any disturbance to the adrenal glands on either the left or right sides. All rats were given buprenorphine (0.3mg/kg, subcutaneously) post-surgery to reduce pain.

Recovery and Additional Mating

Rats were monitored until they regained consciousness. The rats were housed in individual cages for 10-14 days, until the rats were fully recovered, and then placed with a male. The males were removed after 18-20 days, prior to the birth of the pups at the end of the 21 day gestation period, to prevent the female from getting pregnant while nursing the litter. Once the breeding males were removed for the second time, the rat cages were monitored for a second litter of pups. Once born, pups remained undisturbed with the mother for four weeks, to reduce the likelihood of cannibalism of the pups. At four weeks of age, the offspring were weaned and sexed. Four male offspring were arbitrarily selected and separated into cages as potential study rats. The remaining offspring were euthanized. At the time of weaning, the maternal SHR were euthanized by rapid decapitation, and trunk blood was collected in both heparinized and clotting tubes. Maternal hematocrit, weight, and stress level were recorded. Serum was separated and frozen for later analysis of plasma aldosterone and corticosterone. At 11-13 weeks of age, two of the four male offspring were arbitrarily selected from each second litter and implanted with a remote monitoring device to record cardiovascular parameters. The extra males were used if an

implant surgery was unsuccessful. Once two pups were successfully implanted, any remaining pups from that litter were euthanized by CO_2 inhalation.

PA-C40 Implantation

At 11-13 weeks of age, the SHR male offspring were surgically implanted with a remote monitoring device used to collect data on blood pressure, heart rate, and activity level. The remote blood pressure monitoring device (PA-C40) consists of a transmitter and catheter. The implants remained in the SHR male offspring for four weeks to allow for four weeks of data collection.

The implantation surgery was performed as follows. The sterilized BP monitoring device (PA-C40, Data Sciences International, St. Paul, MN) was placed into a beaker of sterile saline. The rat was shaved to prevent hair from entering the sterile surface surrounding the incision. The shaved area included both the legs and the caudal half of the ventral abdomen. A 2-3 cm inguinal incision was made perpendicular to the femoral groove. Blunt dissection was used to part the fat and connective tissue surrounding the femoral artery. The most proximal section of the femoral artery was isolated.

Three sutures were preplaced around the isolated section of the femoral artery. The proximal suture was used to temporarily occlude blood flow while the catheter was inserted. The middle suture was used to seal the artery around the catheter stem. The distal occlusion suture was used to permanently ligate the downstream artery. Once the sutures were preplaced, a secure knot was tied around the downstream suture. The catheter introducer consisted of a 22 gauge injection needle with the tip bent 90˚ away from the bevel to allow for the open part of the

bevel to face down when the needle was held vertically. Tension was applied to the downstream occlusion suture and it was anchored it to the sterile work area using tape. A heavy hemostat was then applied to the upstream occlusion suture to produce tension and occlude the blood flow. The middle suture was then loosely tied, and would be tightened to secure the catheter in place once it was introduced into the vessel. The transmitter catheter was then placed in close proximity to the incision site. The pointed bevel on the bent needle was inserted into the lumen of the femoral artery just proximal to the downstream ligation suture, and the catheter was fed into the vessel lumen. The proximal suture was then released to allow the catheter to be gently inserted further into the artery until approximately 1.5 inches of catheter remained outside. This insured the tip of the catheter was seated properly in the aorta. The middle occlusion suture was repositioned over the artery and catheter, and tied into a knot. The upstream occlusion suture was tied around the artery and catheter as well. The loose ends of the downstream occlusion suture were tied around the catheter stem.

After the catheter was secured and knotted, blunt dissection was used to tunnel under the skin on the ipsilateral side of the rat to create a pocket for placement of the transmitter. The pocket was made cranial enough not to interfere with the hind leg. The tunnel was then irrigated with sterile saline prior to the insertion of the transmitter. The transmitter was inserted battery end first into the opening, making sure the skin was not excessively tight over the transmitter body. A suture was then placed to anchor the skin to the body on the inside of the animal's flank, which prevented the transmitter from migrating back to the inguinal region. Once the transmitter was placed correctly, the skin was closed with sterile monofilament nylon suture (4-0; 3/8 circle cutting needle; Henry Shein). The rat was injected with buprenorphine (0.3 mg/kg subcutaneously) and placed in a cage for recovery and data collection.

The remote monitoring device sends blood pressure data via radio signal to a receiver connected to a computer. The receiver was placed directly under the rat's cage. The computer was programmed to record 15-second segments, once an hour for 48 successive hours during each of the 4 weeks of data collection, which began within a few days of implantation. Batteries were shut off during the days the rats were not monitored to conserve battery life. After the fourth week of monitoring, rats were euthanized by rapid decapitation and trunk blood was collected. Hematocrit was measured, and serum was separated and frozen for later analysis of plasma aldosterone and corticosterone. The implant devices were removed, cleaned, re-sterilized, and implanted into the next rat.

Euthanasia by Rapid Decapitation

Rapid decapitation is the only well accepted means available to collect blood prior to minimize stress-initiated elevation of plasma aldosterone and corticosterone levels. SHR were quickly coaxed into a plastic "restraining bag" (Harvard Apparatus), and decapitated, using a guillotine, within 2 minutes of restraint. Trunk blood was collected into a serum separator tube and a heparinized test tube using funnels. The small heparinized blood sample was used for hematocrit analysis. Hematocrit analysis was used to indicate the proportion of blood made up of red blood cells to determine if the animals had an excess destruction of red blood cells, or a reduction in red blood cells. Hematocrit analysis also indicates if rats were dehydrated at the time of collection. Serum was stored at -80°C until hormone analysis.

RIA of Plasma Hormones

Radioimmunoassay kits for aldosterone and corticosterone were obtained from Siemens Healthcare Diagnostics (Coat-A-Count Corticosterone, Catalog number TKRC1, DPC). Assay procedures followed the manufacturer's directions (Appendix). All samples were assayed at one time to eliminate inter-assay variability.

Data Collection and Analysis

The PA-C40 transmitter measured cardiovascular parameters and activity in SHR offspring. The transmitter consisted of a pressure catheter that transfers pressure from the catheter to a pressure sensor in the transmitter body. The implanted device was turned on or off by waiving a magnet over the outside of the rat. Once the battery implant was turned on, the remote monitor began to collect data. Data were collected approximately 2 ½ days per week during which the computer was set to take a 10 second sample each hour. Data were collected for four weeks following implantation and included the following: systolic (mmHg), diastolic (mmHg), and mean blood pressure (mmHg), heart rate (beats per minute), and activity level. Activity level was determined from rat movement patterns. When the rat's hind limb moved (the side the catheter was inserted), the catheter and transmitter moved as well. The activity sensed by the transmitter was sent to the receiver and was designated a relative number corresponding to the movement pattern. The data were recorded using the Dataquest A.R.T. software package with factory supplied calibration values (Data Science International).

Systolic, diastolic, and pulse pressure, mean arterial pressure, heart rate, and activity level

were compared using a one way ANOVA to evaluate same age data points between freeze and sham groups. The data were sorted by activity first with the following relative designations: low (0-2), moderate (2-4), and high activity (4-8). A two-way ANOVA was used to compare data points over time between groups. Tukey/Kramer post hocs tests were performed where significance was found.

A continuous scatterplot with linear regression analysis compared activity level versus mean pressure for each week of the four week data collection period. The slopes and elevations of the regression lines from the sham and frozen groups were compared using a two-way ANOVA.

A t-test was used to compare plasma corticosterone and aldosterone levels between groups. Additionally, maternal and offspring hematocrits were compared using a t-test in sham and frozen treatments. All differences were considered significant at $p < 0.05$.

Chapter IV

Results

General

No significant age/weight differences were found in maternal rats subjected to either the sham or freeze procedure (73.6±1.8 vs. 71.9±2.5 days) at the time of adrenal freezing. However; maternal frozen rats were heavier on average at the time of surgery but, not significantly (206±6.7 vs. 226±10.7 grams; P=0.09). The SHR pups were then separated from their mothers after 4 weeks of weaning. No significant difference was found between sham and frozen maternal weight at the time of weaning (255±13 vs. 263±14 grams). Also, no significant difference was observed when calculating the difference between maternal weight at the time of surgery and maternal weight at the time of weaning (47.9±7.5 vs. 37.4±8.4 grams) (Table 1).

A total of 30 pups from sham (n=16) and adrenal frozen (n=14) mothers were surgically implanted with a remote monitoring device that collected data on heart rate, activity level, systolic, diastolic, pulse, and mean arterial blood pressure. When comparing timeline data, no differences were found in the time span between any of the following: time between the maternal sham/freeze date and offspring date of birth (37.6±0.5 vs. 37.1±1.0 days), between the offspring date of birth and implant date (84.6±1.6 vs. 83.4±1.3 days), or between the implant date and the time of first data collection (3.05±0.9 vs. 3.00±0.8 days) with sham and adrenal frozen values reported respectively (Table 1).

Pup weights at the time of implantation and euthanasia were not significantly different in sham versus adrenal frozen groups, although the offspring of adrenal frozen rats were slightly

heavier (280±5 vs. 294±7 grams at implantation; P=0.082 and 334±1 vs. 348±6 grams at euthanasia; P=0.088). However, the difference between implantation weight and euthanasia weight (weight gain) was similar (53.9±3.3 vs. 53.8±1.0 grams) (Table 1).

Hematocrit Levels

Maternal and offspring hematocrit levels were measured after rapid decapitation and trunk blood collection. Hematocrit levels are recorded as the percent volume of blood that is composed of cells. No significant differences in hematocrit levels were observed between sham and adrenal frozen groups in the maternal rats (42.8±1.8 vs. 44.7±3.0 %, respectively), or in the offspring (46.2±1.4 vs. 45.9±1.5 %) (Figure 1, Table 2).

Plasma Hormone Levels

Two rat serum samples were removed from the hormone analysis (one from each group) based on laboratory notebook records indicating elevated stress levels at the time of decapitation and markedly elevated plasma corticosterone and aldosterone values. Plasma corticosterone levels were not significantly different between sham and adrenal frozen mothers (156±39 vs. 167±74 ng/dL). Pups from the adrenal frozen mothers had a reduced level of corticosterone compared to pups from sham mothers (57.8±20 vs. 140±41 ng/dL; P=0.079) (Figure 2), but this difference was not significant. Plasma aldosterone was significantly lower in the adrenal frozen mothers (2.97±0.8 vs. 0.54±0.4 ng/dL) versus the sham (P=0.013). Plasma aldosterone levels were not different in the pups between the two groups (6.99±2.5 vs. 3.87±2.1 ng/dL) (Figure 3),

(P=0.332) (Figure 2, Table 3)

Cardiovascular Parameters

SHR offspring were implanted with a PA-C40 remote monitoring device to record cardiovascular parameters including heart rate, activity level, systolic, diastolic, pulse, and mean arterial blood pressure. The pulse pressure, mean arterial pressure, systolic pressure, and diastolic pressure were averaged for each week for each rat at low (0-2), moderate (2-4), and high (4+) activity levels (Figures 3, 4, 5; Tables 4, 5, 6). Group means for each activity level and week was calculated from the individual rat mean values, and levels were compared between offspring from sham and adrenal frozen mothers.

No significant differences were observed in heart rate, systolic, pulse, diastolic, and mean arterial blood pressure in low, moderate, and high activity levels in any of the four weeks between sham and adrenal frozen offspring. A trend was visible in that systolic, diastolic, and mean arterial pressures were consistently higher in the sham group than in the adrenal frozen group, across all ages and activity levels. Within group differences were observed between low, moderate, and high activity levels but no significant differences were found between groups at each activity level (Figures 3, 4, 5; Tables 4, 5, 6).

Regression Data on Blood Pressure versus Activity Level

A scatterplot with a regression line compared activity level versus mean pressure for each of the four weeks of data collection (Figures 5, 6, 7). The slopes and elevations of the regression lines from the sham and frozen groups were compared using a two-way ANOVA.

When plotted against activity, there was no was no significant difference in pulse pressure values comparing slopes between groups during any weeks (Figure 5). Heart rate showed similar results for weeks 1, 2, and 3, but the regression slope for week 4 was significantly more shallow in the adrenal frozen group that in the sham ($P=0.016$) (Figure 6).

When plotted against activity, mean arterial pressure had a significantly more shallow slope during weeks 2 and 3, ($P<0.001$; $P=0.049$), and had a lower overall elevation during weeks 1 and 4 ($P=0.033$; $P<0.001$) (Figure 7).

Chapter V

Discussion

Overview

A significant reduction in maternal aldosterone was observed in SHR subjected to unilateral adrenalectomy and a contralateral zona glomerulosa freezing procedure indicating that the fetuses of the adrenal freeze procedure had compromised aldosterone levels during gestation. Maternal corticosterone levels did not differ significantly between the two groups. Male SHR pups in the two groups did not demonstrate significant differences in plasma aldosterone or corticosterone levels. The maternal adrenal freezing procedure was found to significantly reduce the slope of the mean pressure of male offspring when plotted against activity level during weeks 2 and 3 of the study. Mean arterial pressure of offspring in the adrenal frozen group was significantly lower than sham offspring during week 4. A similar trend was visible during week 1 but, this was not physiologically significant. Pulse pressure was significantly lower (overall) in most weeks, but the mean difference was only 1 mm, which is not physiological significant. Heart rate also was significantly lower most weeks, but the difference was only 5 bpm, which is not physiologically significant given that heart rates were greater than 300 bpm. Heart rate followed a similar trend as mean pressure, showing a shallower slope when plotted against activity. However, this was only significant during week 4.

Plasma Hormone Levels

The adrenal freeze procedure was found to significantly lower maternal plasma aldosterone concentrations but did not alter corticosterone concentrations in maternal SHR ($P=0.013$ and $P=0.882$, respectively). Although these values were obtained four weeks post parturition, prior studies in this lab have shown that adrenal freezing of maternal rats prior to mating lowers aldosterone but not corticosterone when measured on day 21 of gestation. These results suggest that the adrenal freeze procedure successfully altered zona glomerulosa cell function without compromising hormone secretion in the other layers of the adrenal cortex. Offspring concentrations of plasma aldosterone and corticosterone were not significantly different between sham and frozen groups.

The reduction in maternal aldosterone synthesis will affect the supply of aldosterone to the fetus because maternal circulation of aldosterone and other steroid hormones can cross into the fetus through the placenta (65). Since zona glomerulosa cells are not present in the fetus until gestational day 16, maternal aldosterone secretion is important for fetal development. Immunohistochemistry and in situ hybridization was used to determine the expression of P450aldo in the adrenal cortex of rats (66). P450aldo, a chemical marker indicating the production of aldosterone synthase, was present in small amounts in the adrenal cortex at day 16 of gestation. P450aldo distribution changed by gestational day 19, into a discontinuous ring of cells 4- to 5-cells wide under the capsule, forming the functional zona glomerulosa (66). Therefore, alterations in the maternal adrenal hormones would have an effect on the hormone levels in the developing fetus until the zona glomerulosa is fully developed until at least gestational day 18-19 (18, 66).

Growth

The reduction in aldosterone synthesis in maternal frozen rats did not appear to affect offspring growth, since offspring weights at 11-13 weeks of age were not significantly different between groups. Although it is possible that offspring weights were reduced at birth, prior studies in this lab have found no difference in fetal weights between maternal adrenal frozen and control groups at day 21 of gestation. Aldosterone plays a role in blood volume expansion during pregnancy (67, 68), and lower aldosterone would be expected to reduce maternal blood volume which could potentially affect fetal growth. Furthermore, aldosterone appears necessary for normal placental development since aldosterone deficient mice produced fewer and smaller offspring (51, 52). The potential for reduced aldosterone to alter fetal development has been demonstrated in a study which observed the effect on cultured embryos of blocking adrenocortical hormones with ZK 91587, a mineralocorticoid receptor antagonist (69). When receptor activity was obstructed, damaging effects on embryonic development occurred. The effect of the mineralocorticoid antagonist was found to be reversed by the direct administration of aldosterone to the embryos by infusion. Adverse effects on rat embryos observed included reduced total length, somite number, compromised communication between the vitelline and umbilical cord, impaired vascularization of the vitelline sac, and reduced embryo curvature (69). The absence of a fetal growth effect in the present study may be due to aldosterone not being lowered enough to significantly decrease maternal fluid and blood volume, or to impair placental development. It is also possible that other hormones such as corticosterone may contribute to the expansion of blood volume that occurs during gestation (67, 70).

Few studies have examined lowered aldosterone during pregnancy, but several have looked at altered maternal sodium intake. Sodium intake has a profound effect on aldosterone,

through its influence on the renin-angiotensin aldosterone system (RAAS). Maternal low sodium diets have been shown to lower maternal blood volume, and activate the RAAS, increasing maternal and fetal aldosterone levels (71). Maternal high sodium diets expand maternal blood volume and suppress the RAAS, reducing plasma aldosterone levels (72). Thus alterations in maternal sodium intake could produce blood volume effects similar to those produced by alterations in aldosterone (71).

Maternal sodium alterations have been shown to affect fetal development. Deloof et al. (2000) studied the effects of high maternal sodium intake during late gestation on maternal and fetal hormone secretion and body growth in WKY rats (72). The results of the study found no change in maternal weight when sodium intake was altered, although fetal weight did increase in response to the high salt diet. The increase in fetal body weight was attributed to the increase in water retention associated with a sodium increase (72), however alterations in the RAAS were not ruled out as a mechanism. Other studies have found that a low sodium diet in maternal rats reduced plasma volume, increased aldosterone and induced growth restriction in the fetuses (73, 74, 75). However, by 14 days of age, pups were of normal size (73, 75). A high sodium diet would result in low maternal aldosterone, similar to our study, while a low sodium diet would raise maternal aldosterone. Deloof found that the high salt diet caused nearly a 50% decrease in plasma aldosterone in pregnant rats and fetal rats (72). The decrease in aldosterone from the high salt diet is due to the reduction in the maternal renin-angiotensin-aldosterone system, which reduces aldosterone secretion. While the adrenal freeze procedure may produce aldosterone concentrations that mirror the results of a high sodium diet, the changes are not attributed to alterations in renin and angiotensin. Furthermore, the rats in the current study would not likely have water retention, but would more likely have volume depletion.

Hematocrit

No significant differences in hematocrits were observed between sham and adrenal frozen groups in the maternal rats (42.83±1.79 vs. 44.71±3.00 %, respectively), or in the offspring (46.18±1.41 vs. 45.91±1.48 %). Deloof et al (2000) found no change in fetal hematocrit levels of maternal WKY given a high sodium diet despite changes in plasma aldosterone concentrations and sodium intake (72). In the current study, adrenal freezing lowered plasma aldosterone levels, and may have produced lower maternal blood volumes. However, a lower blood volume will not necessarily influence hematocrit levels. If the concentration of red blood cells remains constant, hematocrit levels will remain normal regardless of the total blood volume (76).

Mean Arterial Pressure

Blood pressure did not increase as much with activity in the offspring of the adrenal frozen group versus the control group for weeks 2 and 3, and was overall lower during week 4. Blood pressure is a function of heart rate, stroke volume, and vascular resistance. Heart rate also showed a lower rate of increase with activity in the adrenal frozen group than in the control group. Pulse pressure, known to be related to stroke volume, had an overall slight decrease in the adrenal frozen group, but this change was small (1 mmHg). Although pulse pressure and heart rate changes were not significant individually, together they may explain, in part, the observed changes in mean arterial pressure. An increase in sympathetic nervous system activity occurs with an increase in activity, producing the higher heart rates, stroke volumes, and mean arterial blood pressures observed during exercise. A lesser increase in sympathetic stimulation

with activity could explain the current results (77, 78, 79). During the 4th week, the overall mean pressure was lowered, but the mean arterial pressure response to activity returned to normal in the adrenal frozen group.

A reduction in blood volume could explain the lower blood pressure during week 4. However, if the adrenal frozen group offspring had been volume depleted, a smaller pulse pressure and faster heart rate would have been expected, but this did not occur. Therefore, it is unlikely that volume depletion caused this reduction in mean arterial pressure. Both pulse pressure and heart rate tended to be lower during week 4, suggesting an overall lower level of sympathetic nervous system stimulation (77, 78, 79).

The mechanism underlying the alterations in the cardiovascular system of the offspring of the adrenal frozen rats is unclear. Reduced maternal plasma volume could have occurred as a result of low aldosterone. A study in sheep found fetal arterial pressure to be significantly related to maternal plasma volume, which was altered by lowering aldosterone or cortisol (67). The authors concluded that maternal volume expansion may be necessary for normal fetal blood pressure, glucose levels, and blood oxygen tension, and that normal adrenal secretion is necessary for maternal and fetal homeostasis. Although fetal blood pressure was reduced with low aldosterone, blood pressure was not monitored in the lambs after birth.

Alterations in maternal sodium diet can affect maternal plasma or blood volume, and alter plasma aldosterone levels. Several studies have found that altered maternal sodium can affect offspring cardiovascular homeostasis. High sodium maternal diets have been shown to result in higher mean arterial blood pressure in rat offspring (48, 70) and increase the responsiveness of the RAAS (75). Koleganova et al (2011) found that both high and a low maternal salt intake can

raise mean arterial blood pressure in the offspring. High sodium diets also increase the sympathetic activation in response to stress (48, 81). None of the alterations in maternal sodium intake appeared to confer the beneficial effects on the offspring cardiovascular system that were observed with the reduced maternal aldosterone of the present study. Low sodium diets likely reduce plasma or blood volume in the maternal rat, as would be expected in the current low aldosterone model. High sodium diets would likely suppress maternal aldosterone, again similar to the current study. However, the combination of low aldosterone and reduced blood volume is not likely observed with either diet, and this may explain the different effects noted in the present study.

Conclusion

This study was designed to investigate in SHR, the effects of maternal aldosterone suppression on the development of hypertension in the offspring. The freeze procedure, right adrenalectomy combined with cryodestruction of the zona glomerulosa of the left adrenal gland, successfully reduced maternal aldosterone concentrations and influenced fetal development leading to changes in adult offspring cardiovascular function. The adult offspring of the adrenal frozen group were found to have an attenuated mean pressure response to activity during weeks 2 and 3, and an overall lower mean pressure during week 4. Similar patterns in heart rate suggest that an attenuation of sympathetic activation with increased activity was likely responsible for the group differences. This fetal programming effect has not been observed with other maternal manipulations involving aldosterone.

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Appendix

The serum samples were taken out of the freezer (plasma frozen at -80°C) and thawed to room temperature overnight. Four uncoated polypropylene plain tubes (12x75mm) were labeled in duplicate for non-specific binding and total count. Corticosterone Ab-coated tubes A through H were labeled in duplicate for the calibrators used to calculate a standard curve. Duplicate tubes were also labeled for each rat serum sample. Samples were pipetted into the bottom of the tubes. 50µL of zero calibrator A was pipetted into A and non-serum binding (NSB) tubes. In addition, 50µL of the each calibrator (standard) was pipetted into tubes B through H, and 50µL of each serum sample was pipetted into the appropriate tube. 1.0mL of ^{125}I Rat Corticosterone was added to each tube, and the tubes were vortexed briefly to mix. The tubes were then incubated for 2 hours at room temperature allowing for the antibodies to bind, and then all tubes except the T (total counts) tubes were decanted. In order to remove any visible contents remaining in the tube, all tubes (except for the T tubes) were turned over and struck against absorbent paper. The samples and duplicates were then placed into a gamma counter (Ludum Measurements, Model 2600) for 1 minute to read tube radioactivity. The CPM (counts per minute) was recorded for each tube on the corticosterone assay data sheet. Duplicate counts were averaged. The results from the calibration samples were organized into a standard logit-log curve, and an equation was calculated and used to determine plasma corticosterone levels from the sample counts.

The radioimmunoassay procedure for rat aldosterone (Coat-A-Count Aldosterone, Catalog number TKAL1,DPC) followed a similar procedure to that of rat corticosterone. Aldosterone Ab-Coated tubes were labeled in duplicate A through G for the calibrators. Plain plastic tubes were labeled with NSB and T, and aldosterone Ab-coated tubes were labeled in duplicate for each rat serum. 200µL of calibrator A was added to the NSB tubes, 200µL of each

calibrator was added to tubes labeled A through G, and 200µL of each serum sample were pipetted according to their respective rat identification tags. 1.0mL of ^{125}I Rat Aldosterone was also added to each tube, which was then vortexed briefly. The tubes incubated for 18 hours at room temperature to allow for the antibodies to bind, were decanted, and then were read for 1 minute in the gamma counter. Recorded CPM of each sample and its duplicate were averaged, and a standard logit-log curve and equation were calculated. The equation was used to determine rat serum aldosterone levels.

Table 1. Weights and timeline data from maternal and offspring sham (n=10; n=16) and adrenal frozen (n=8; n=14) SHR.

General Weight and Timeline Statistics			
	Sham	**Frozen**	**P-value**
Maternal weight at the time of surgery	206±7	226±11	0.092
Maternal weight at euthanasia	255±13	263±14	0.644
Offspring weight at the time of implant	280+5	294±7	0.082
Offspring weight at the time of euthanasia	334±6	348±6	0.088
Time from surgery to birth of pups (days)	38±1	37±1	0.675
Pups age at implant (days)	85±2	83±1	0.588
Implant to start of data collection (days)	3.1±1	3.0±1	0.968

Table 2. Hematocrit values for maternal and offspring SHR for the sham and adrenal frozen groups. Blood was collected four weeks after parturition (approximately 12 weeks after the sham/freeze procedure) in the mothers, and after four weeks of data collection (14-16 weeks of age) in the offspring.

Hematocrit				
	Mothers		Offspring	
	Sham	Freeze	Sham	Freeze
Mean	42.8	44.7	46.2	45.9
SE	1.8	3.0	1.4	1.5

Table 3. Plasma hormone values for maternal and offspring SHR for the sham and adrenal frozen groups are shown below. Blood was collected four weeks after parturition (approximately 12 weeks after the sham/freeze procedure) in the mothers, and after four weeks of data collection (14-16 weeks of age) in the offspring.

Plasma Hormone Levels								
	Aldosterone				Corticosterone			
	Offspring		Mothers		Offspring		Mothers	
	Sham	Freeze	Sham	Freeze	Sham	Freeze	Sham	Freeze
Mean	7.0	3.9	3.0	0.5	140.0	57.8	156.0	167.1
SE	2.5	2.1	0.7	0.4	40.7	20.3	39.3	73.6
P-value	0.3327		0.0128*		0.0793		0.8823	

Table 4. Cardiovascular parameters measured by an implanted remote monitoring device during low activity levels in SHR male offspring of adrenal frozen or sham operated maternal rats. No significant differences were found.

Cardiovascular Parameters: Low Activity								
	Wk 1		Wk 2		Wk 3		Wk 4	
	Sham	Frozen	Sham	Frozen	Sham	Frozen	Sham	Frozen
Pulse P	50.7±2.6	51.6±1.2	55.0±2.0	56.2±1.1	56.5±2.1	55.6±0.9	57.0±2.2	53.2±2.2
Systolic	172±4	171±2	174±3	172±1	178±3	174±2	179±3	175±2
Mean P	146±4	144±2	146±3	143±1	149±3	146±1.5	149±3	147±2
HR	383±7	380±5	363±5	360±4	355±5	358±5	360±6	350±4
Diastolic	121±3	119±2	119±3	116±1	122±3	119±1	122±3	121±2
Activity	5.43±0.2	5.76±0.2	5.39±0.2	5.38±0.2	5.50±0.2	5.26±0.3	5.32±0.2	5.21±0.2

Table 5. Cardiovascular parameters measured by an implanted remote monitoring device during moderate activity levels in SHR male offspring of adrenal frozen or sham operated maternal rats. No significant differences were found.

Cardiovascular Parameters: Moderate Activity								
	Wk 1		Wk 2		Wk 3		Wk 4	
	Sham	Frozen	Sham	Frozen	Sham	Frozen	Sham	Frozen
Pulse P	51.4±2.0	52.0±1.2	54.0±2.1	55.6±1.1	56.2±2.2	54.5±0.8	54.9±2.5	52.2±1.9
Systolic	173±2.5	172±2	171±2	171±2	178±3	172±2	175±3	170±2
Mean P	147±2.1	146±2	143.5±2	143±2	149±2	144±2	147±3	143±2
HR	372±5	371±6	353±5	355±3	354±6	355±6	342±5	348±4
Diastolic	122±2	120±2	117±2	115±1	122±3	118±2	121±3	118±2
Activity	2.79±0.1	3.00±0.1	2.98±0.1	2.85±0.0	2.90±0.1	2.83±0.1	2.90±0.0	2.89±0.1

Table 6. Cardiovascular parameters measured by an implanted remote monitoring device during high activity levels in SHR male offspring of adrenal frozen or sham operated maternal rats. No significant differences were found.

Cardiovascular Parameters: High Activity								
	Wk 1		Wk 2		Wk 3		Wk 4	
	Sham	Frozen	Sham	Frozen	Sham	Frozen	Sham	Frozen
Pulse P	50.7±2.5	51.6±1.2	55.0±2.0	56.2±1.1	56.5±2.1	55.6±0.9	57.0±2.2	53.2±2.2
Systolic	172±4	171±2	174±3	172±1	178±3	174±2	179±3	175±2
Mean P	146±4	144±2	146±3	144±1	149±3	146±2	149±3	147±2
HR	383±7	380±5	363±5	360±4	355±5	358±5	360±6	350±4
Diastolic	121±3	119±2	119±3	116±1	122±3	119±1	122±3	121±2
Activity	5.43±0.2	5.76±0.2	5.39±0.2	5.38±0.2	5.50±0.2	5.26±0.3	5.32±0.2	5.21±0.2

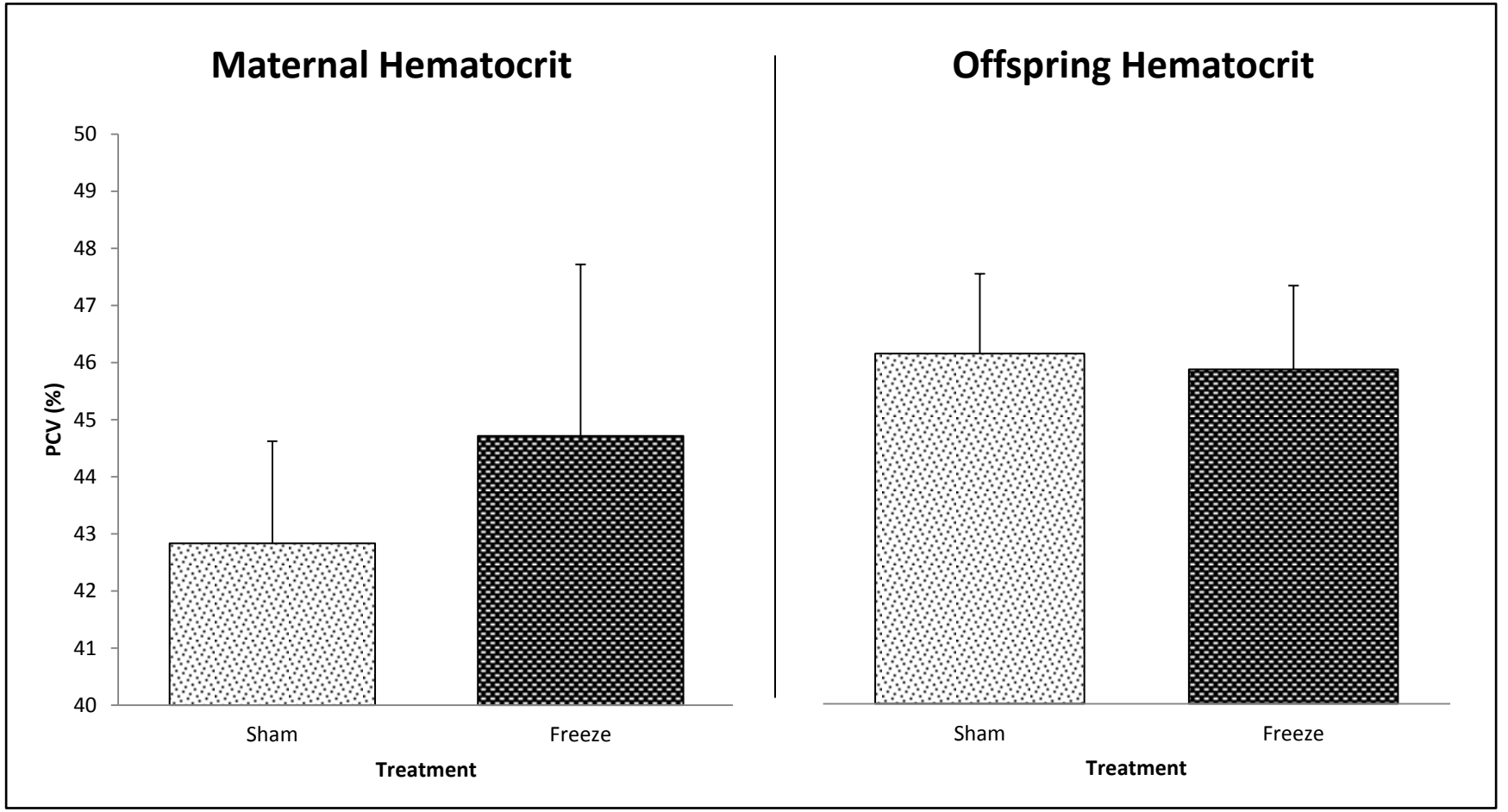

Figure 1. Hematocrit values for SHR mothers and offspring in the adrenal frozen or sham group. Blood was collected four weeks after parturition (approximately 12 weeks after the sham/freeze procedure) in the mothers, and after four weeks of data collection (14-16 weeks of age) in the offspring. No significant differences were observed between groups in either the maternal or offspring SHR.

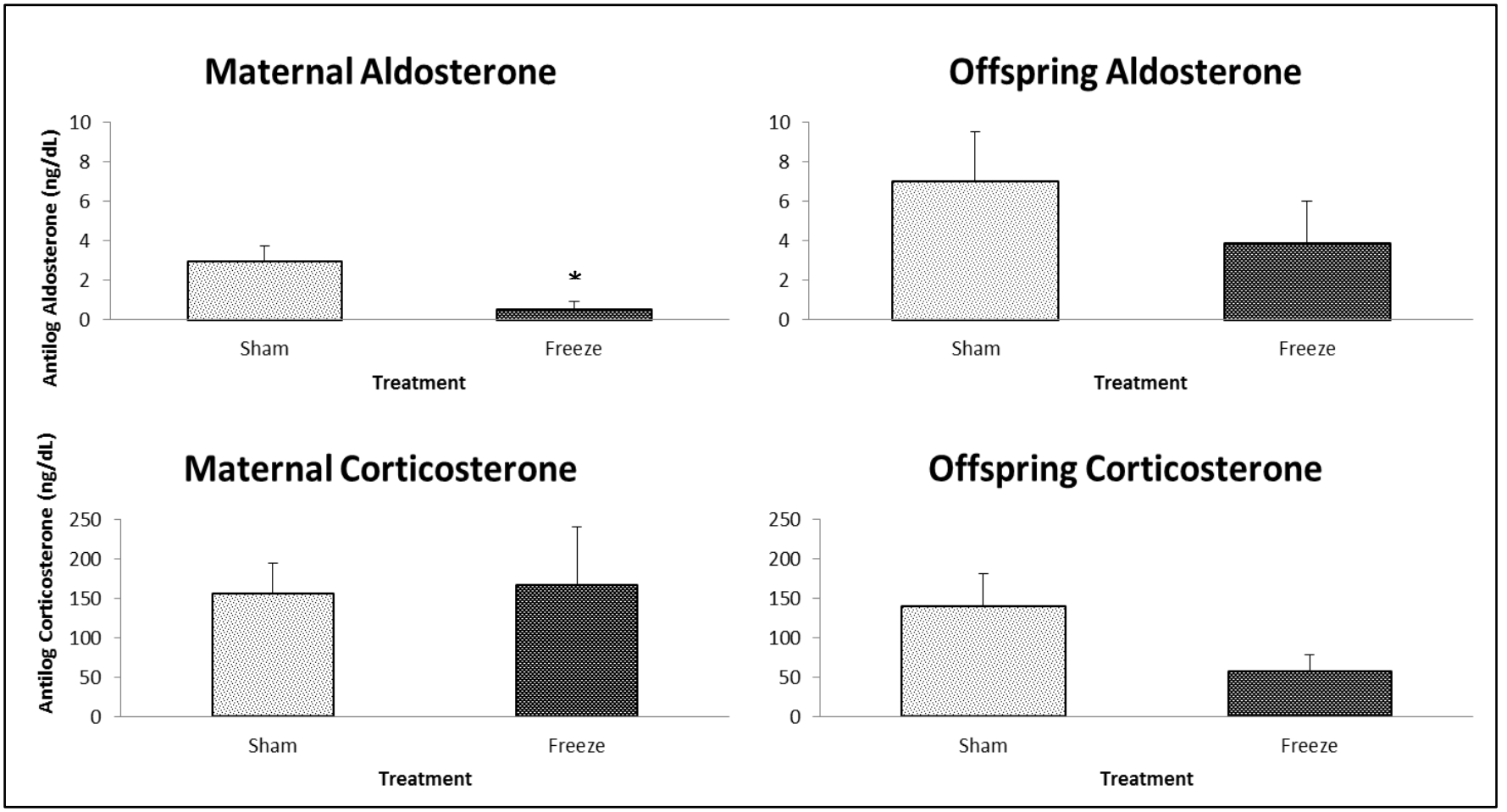

Figure 2. Plasma aldosterone and corticosterone concentrations for both maternal and offspring SHR in both sham and adrenal frozen groups are displayed above. Serum was collected immediately after decapitation. Significant difference in maternal frozen rats compared to sham (*P<0.05).

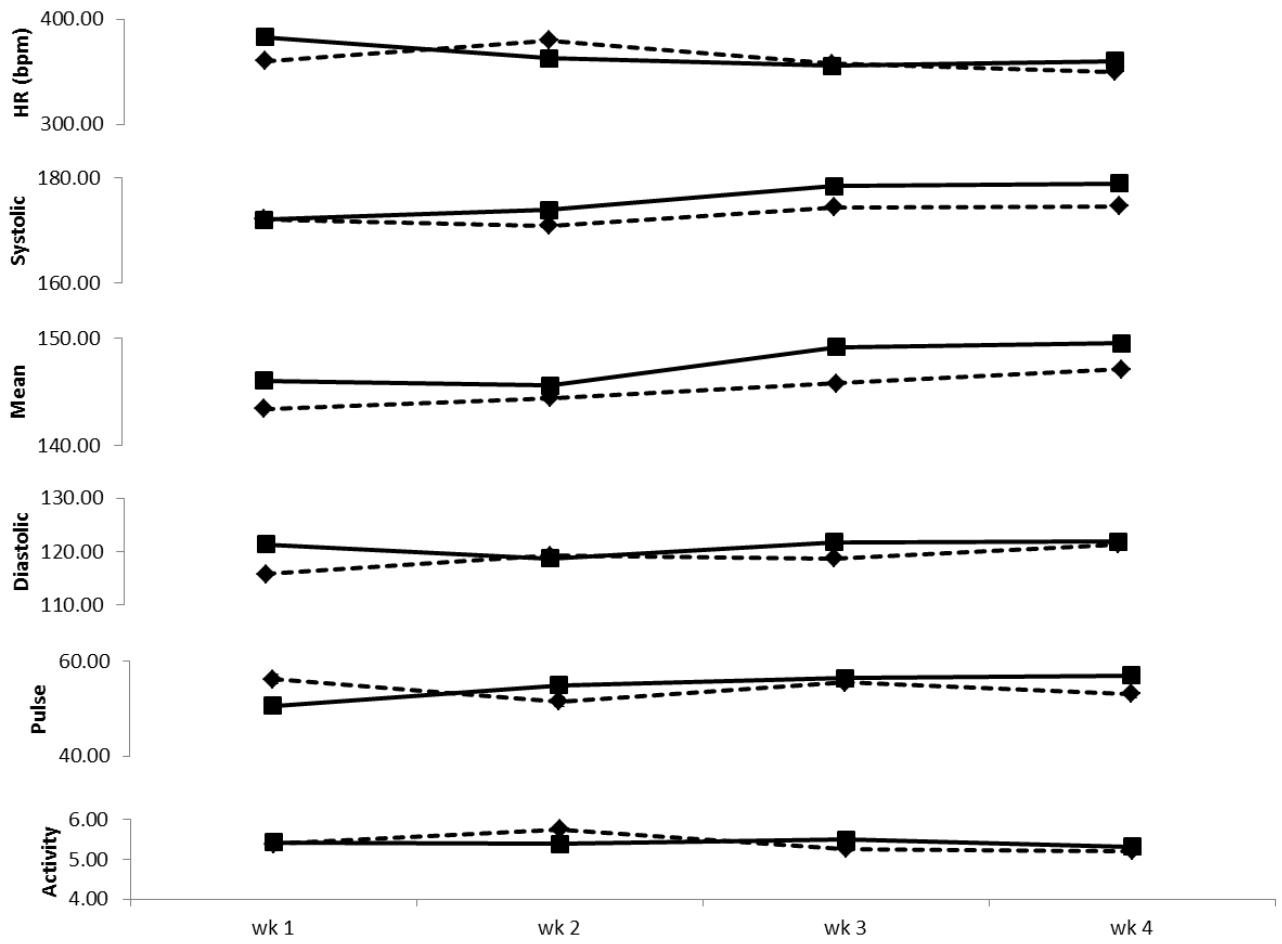

Figure 3. Cardiovascular parameters measured by an implanted remote monitoring device during low activity levels in SHR male offspring of adrenal frozen or sham operated maternal rats. No significant differences were found.

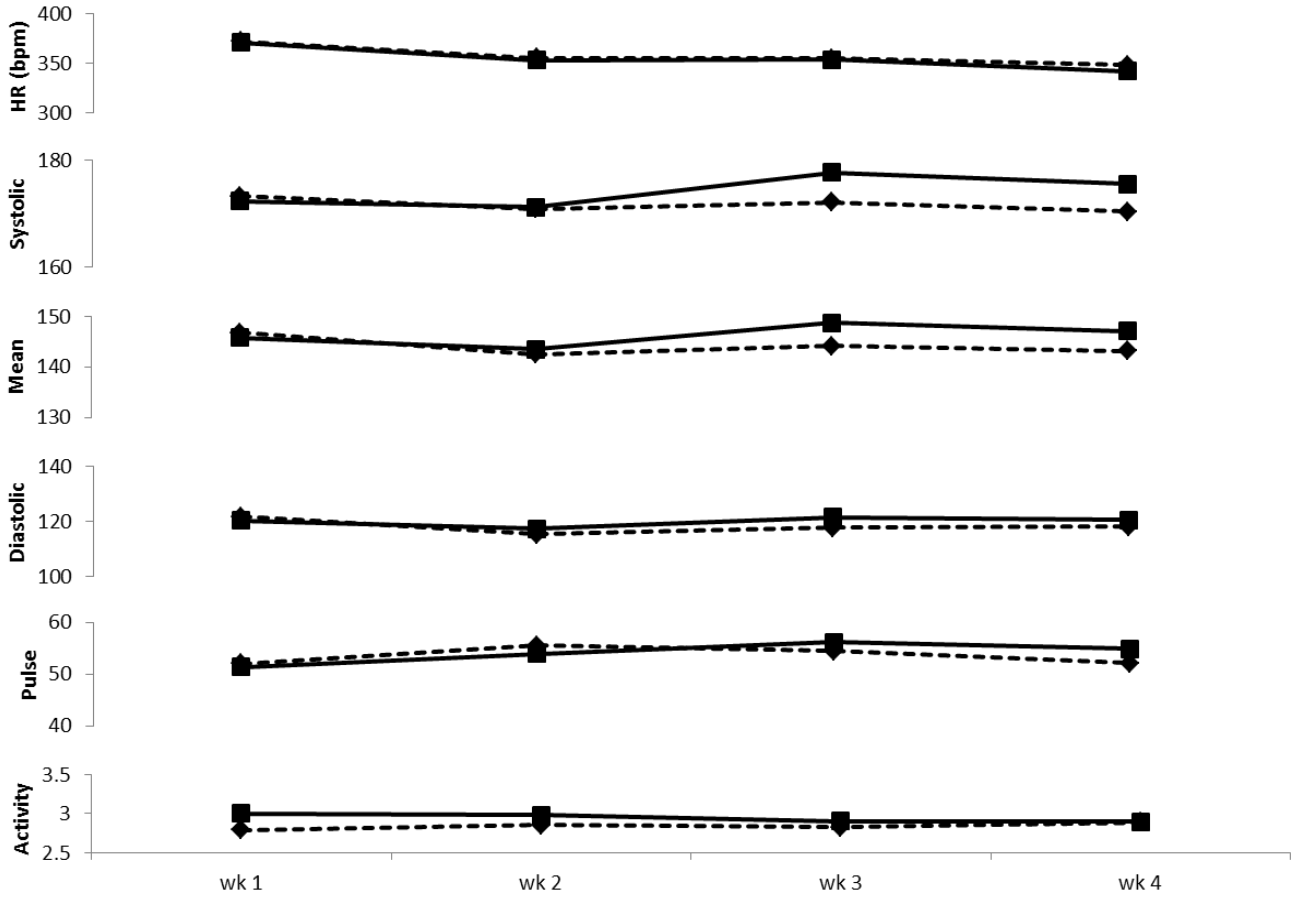

Figure 4. Cardiovascular parameters measured by an implanted remote monitoring device during moderate activity levels in SHR male offspring of adrenal frozen or sham operated maternal rats. No significant differences were found.

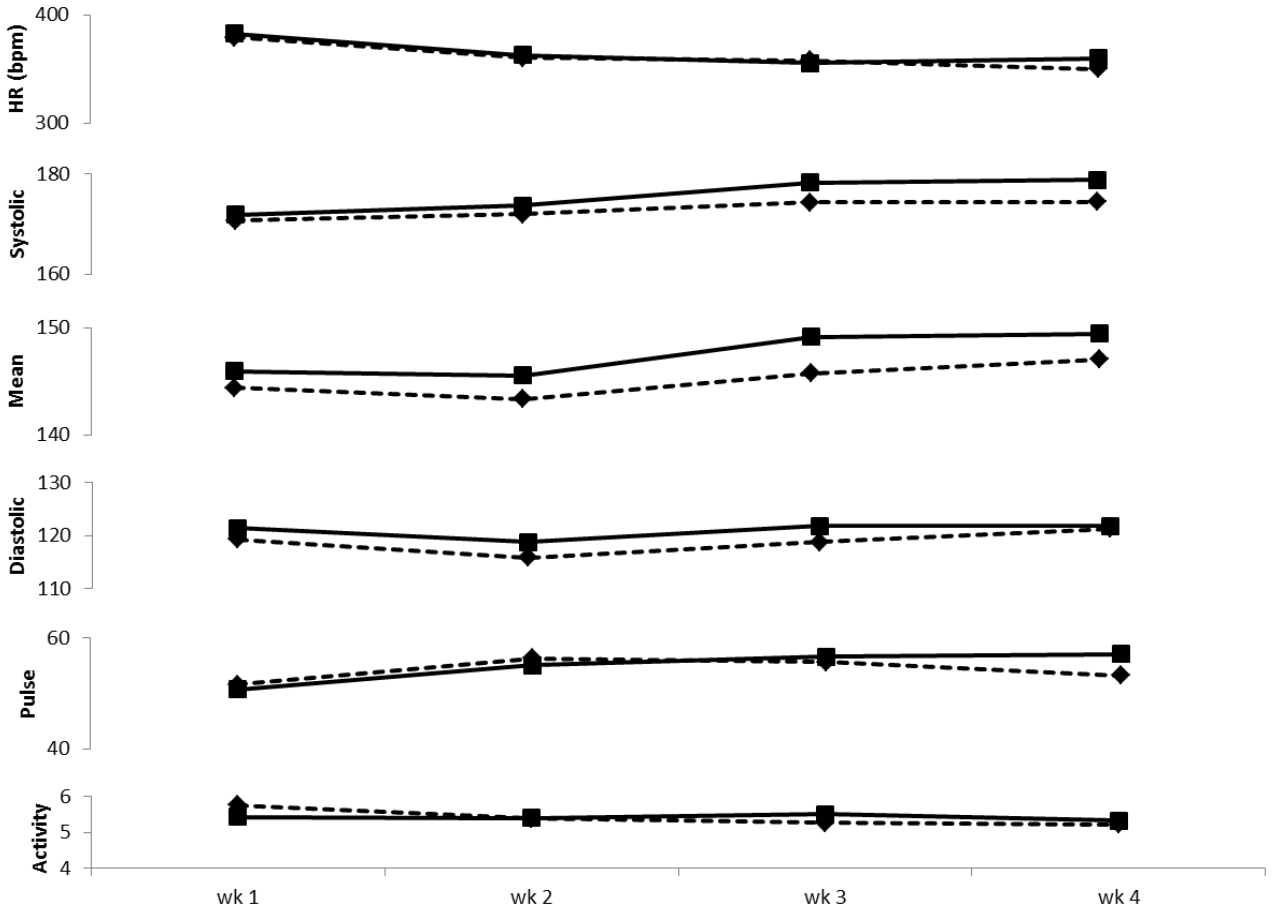

Figure 5. Cardiovascular parameters measured by an implanted remote monitoring device during high activity levels in SHR male offspring of adrenal frozen or sham operated maternal rats. No significant differences were found.

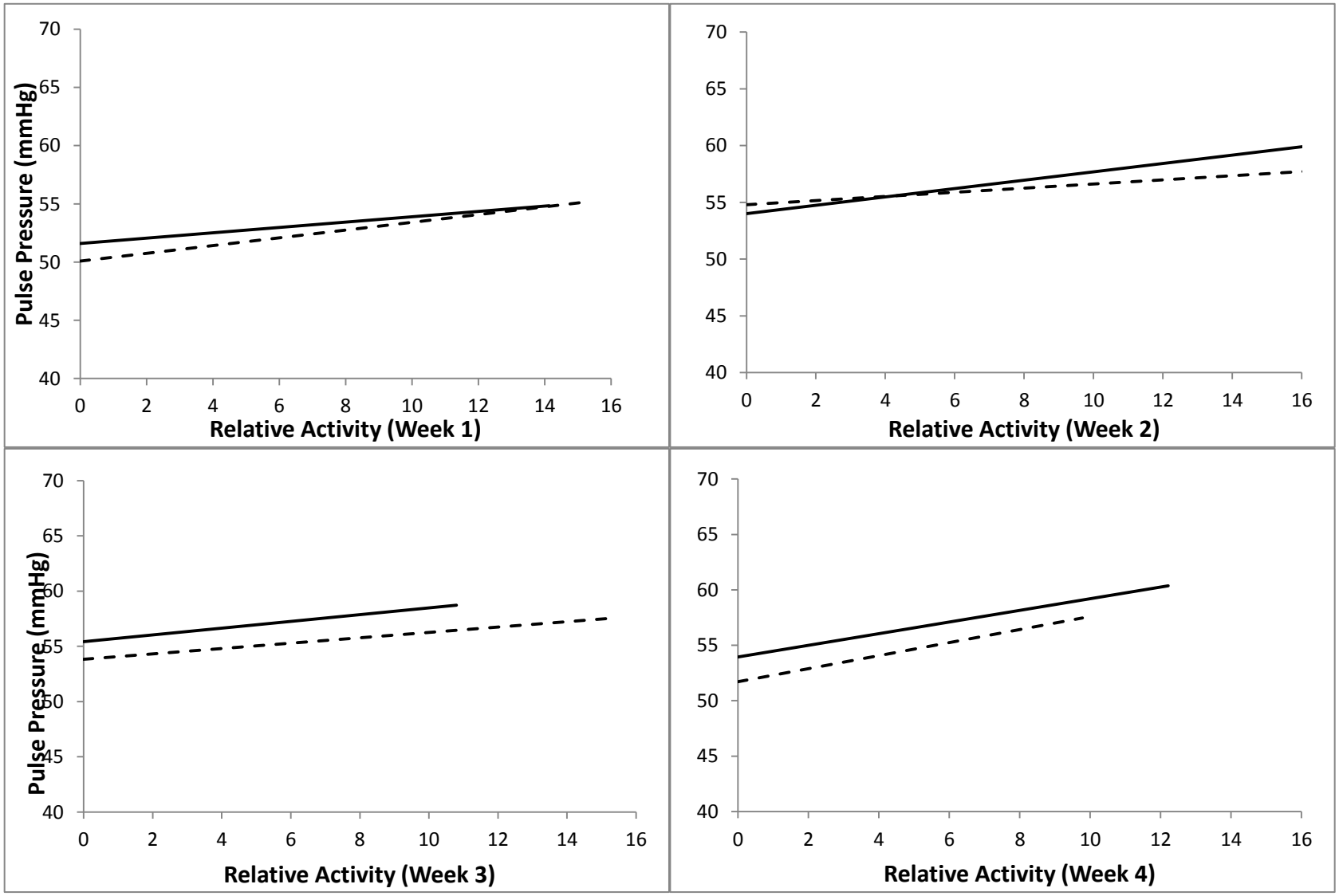

Figure 6. Best fit regression lines for Pulse Pressure versus activity in each of the 4 weeks of data collection in SHR offspring in the sham and adrenal frozen groups. The line slopes were not significantly different.

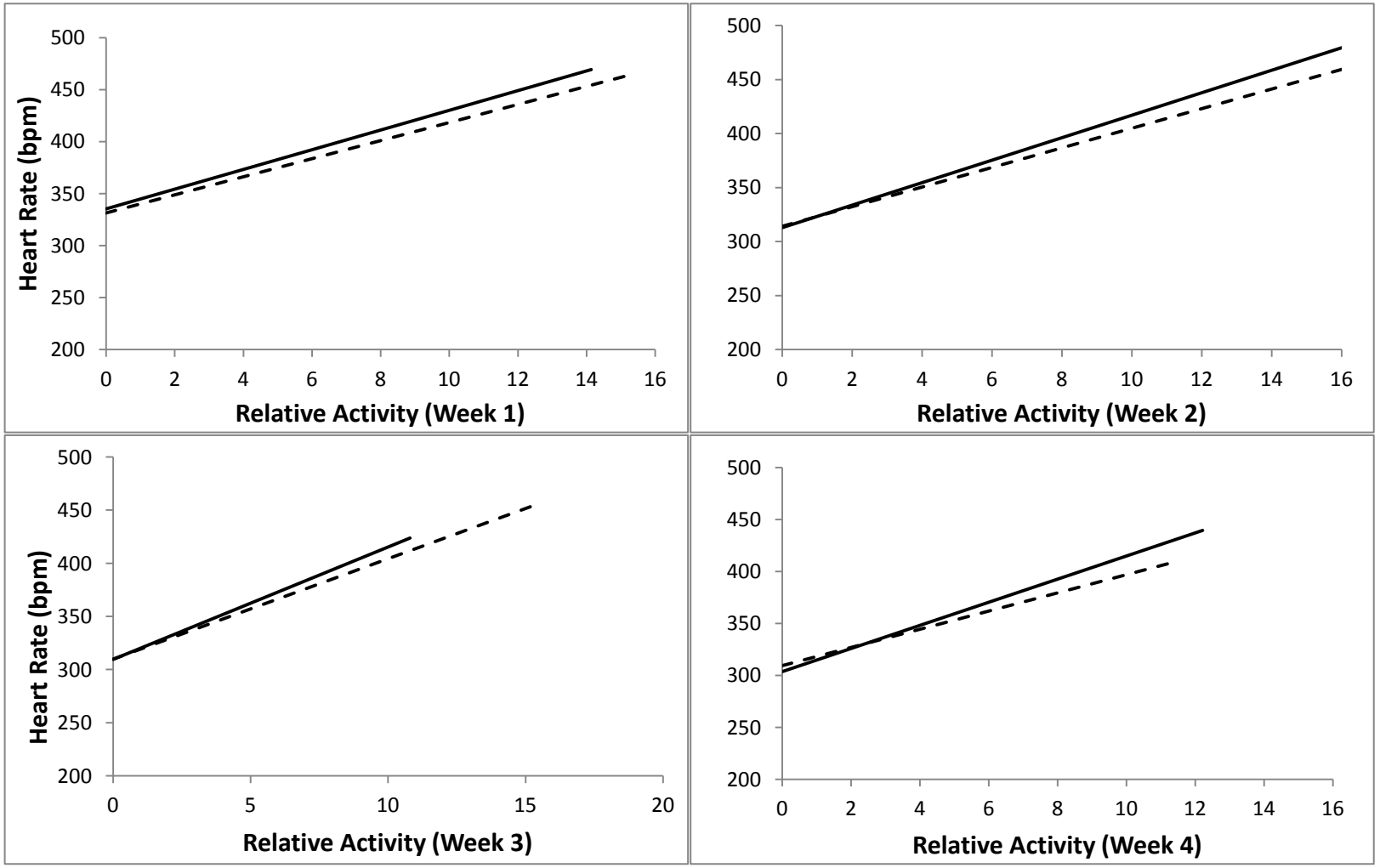

Figure 7. Best fit regression lines for heart versus activity in each of the 4 weeks of data collection in SHR offspring in the sham and adrenal frozen groups. The line slopes were significantly different during week 4.

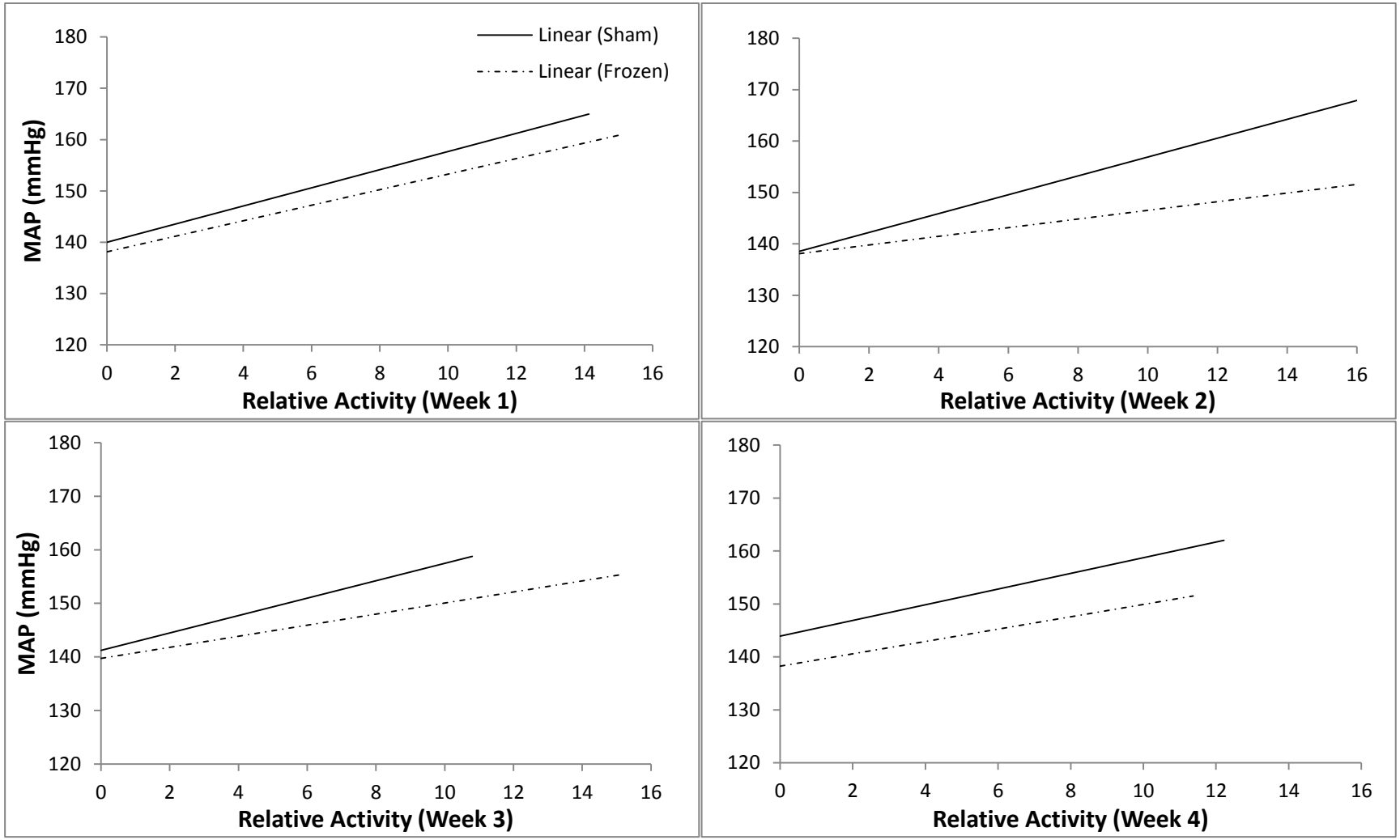

Figure 8. Best fit regression lines for mean arterial pressure (MAP) versus activity in each of the 4 weeks of data collection in SHR offspring in the sham and adrenal frozen groups. The line slopes were significantly different during weeks 2 and 3, and the line for the adrenal frozen group was significantly lower than the sham group during week 4.