UNIVERSITY OF CALGARY

Vascular and renal changes associated with hypertension in adult male rats exposed to maternal smoking during pregnancy

by

Shehr Bano

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled “Vascular and renal changes associated with hypertension in adult male rats exposed to maternal smoking during pregnancy” submitted by Shehr Bano in partial fulfillment of the requirement for the degree of Master of Science in Cardiovascular/Respiratory Sciences.

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Abstract

Objectives: To investigate morphological and histological changes in kidney and variation in blood pressure at adulthood due to prenatal cigarette smokes exposure.

Methods: Eight Wister Kyoto rats in the smoke group were exposed to cigarette smoke while for the sham group equal numbers were exposed to air. Other factors were kept similar between the groups. Blood pressure was recorded on postnatal days 160 and 200 and Kidneys were collected on postnatal days 16 and 200 for microtomy and staining.

Results: Prenatal exposure to smoke significantly reduced kidney weight and body weight on postnatal day 16. Mean ratio between glomerular and cortical fraction areas also reduced significantly, on postnatal days 16 and 200. Mean systolic and diastolic blood pressures significantly increased in the smoke group as compared to the sham group on postnatal day 200.

Conclusion: Prenatal exposure to smoke is significantly associated with histological changes in kidney and elevated blood pressure at adulthood.

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Dedication

This thesis is dedicated to my family

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ACE	Angiotensin Converting Enzyme
CO	Carbon Monoxide
CI	Confidence Interval
DBP	Diastolic Blood Pressure
H&E	Hematoxylin and Eosin
HR	Heart Rate
LBW	Low Birth Weight
MBP	Mean Blood Pressure
μm^2	Micrometer Square
mRNA	Messenger RNA
Manova	Multiple Analysis of Variance
OR	Odd Ratio
PP	Pulse Pressure
ppm	Parts Per Million
SBP	Systolic Blood Pressure
SD	Standard Deviation

Chapter One: Introduction

1.1 Relevance and Significance

During the past two decades, epidemiological and experimental studies have suggested that fetal environment has an important role in fetal organogenesis and development of adulthood diseases (1,2). The role of maternal nutritional deficiency in the development of adulthood diseases has particularly been extensively investigated. These studies provide evidence that maternal nutritional deficiency may cause fetal maladaptive changes that could be attributed to adulthood diseases like hypertension and coronary heart disease (3,4,5,6,7).

Previous studies as well as data from our laboratory suggest that maternal smoking during pregnancy may cause adulthood hypertension, though the underlying cause and mechanism remain unexplained. Literature review however, does not provide ample plausible evidence vis-à-vis certain morphological and histological renal changes that may occur during fetal life as a consequence of exposure to maternal smoking during pregnancy and in turn be the cause of adulthood disease like hypertension. The current study is an effort to fill this gap by exploring whether maladaptive changes in a developing kidney may result from prenatal exposure to smoke and subsequently cause an elevation in blood pressure at adulthood. We planned to program the disease in

an experimental animal model, so that the risk profiles could be explored to protect humans against such diseases (8).

1.2 Research question

The current study is guided by the following research question:

Are the male offspring of the rats exposed to cigarette smoke during pregnancy more likely to develop renovascular changes and hypertension in adulthood as compared to those not exposed to cigarette smoke during pregnancy?

1.3 Objectives

Following were the objectives of the current study:

1.3.1 Primary objective

To explore histological changes in the kidney of an adult male rat secondary to prenatal cigarette smoke exposure during intrauterine life.

1.3.2 Secondary objective

To investigate changes in blood pressure during adulthood in male offspring of rats exposed to cigarette smoke during pregnancy and to correlate such changes with alterations in kidney at adulthood.

1.4 Cigarette smoke

1.4.1 Epidemiological significance

Smoking during pregnancy is a significant public health concern. Maternal smoking increases the risk of spontaneous abortion (9,10,11), low birth weight (12,13), preterm delivery (14,15), sudden infant death syndrome (16,17) and learning and behavioral problems (18,19,20) in offspring. The results from General Social Survey (1996) revealed that Albertan women aged 15 to 24 years were among the heaviest smokers in Canada. It was further determined that 48% of women in this age group were current smokers in 1996 as compared to 29% in 1990. With regard to smoking during pregnancy, Maternal Risk Factor study, in 1999, showed that 26% mothers smoked throughout their pregnancy (21). Canadian Tobacco Use Monitoring Survey, however, revealed that 21% of the population was smoking in 2002. A total of 26% of Canadian women aged 20-44 year became pregnant in the last five years while 11% of these smoked regularly during their most recent pregnancy and 13% said that their spouse smoked regularly at home during their most recent pregnancy (22).

Smoking has also been associated with hypertension and coronary heart disease. In 1997, 22% (26% males and 18% females) of the adult Canadian population was hypertensive. The data from Centre for Disease Control revealed that these figures varied between each of the provinces, ranging from 17% in Alberta to 27% in Newfoundland. Approximately 42% of Canadians with

hypertension were unaware of their condition as narrated by Heart and Stroke Foundation of Canada, in 1999. In 1993, the economic burden of hypertension in Canada was estimated to be $4 billion. The figure that came from the Kidney Foundation of Canada for renal disease was approximately $1.2 billion (23). These effects are more likely either caused by various toxins that may be a constituent of tobacco or smoke.

1.4.2 Contents of cigarette smoke

Cigarette smoke contains more than 4,000 chemicals and some of them, albeit in smaller quantities, have never theless most deleterious effect (24). Cigarette smoke consists of two phases, gas phase and particulate phase. In particulate phase, nicotine, tar, benzene and benzo pyrene are commonly present. The gas phase mainly consists of carbon monoxide, ammonia, dimethylnitrosamine, formaldehyde, hydrogen cyanide and acrolein (25). Nicotine, carbon monoxide and cadmium are among the most harmful constituents in cigarette smoke, while little is known about other constituents (26,27).

1.4.2.1 Nicotine

Nicotine is a naturally occurring alkaloid present in tobacco plant. Chemically, it is an amine consisting of pyridine and pyrrolidine rings. Studies have shown that nicotine crosses some biological membranes including placenta and blood brain barrier. Inhaled nicotine is absorbed in the blood and in the liver it is metabolized and converted into nicotine N'-oxide and cotinine N'-oxide.

Studies have shown that nicotine exposure could also affect gene expression, regulation of hormone secretion and enzyme activities (28).

Nicotine metabolic rate depends upon its blood level. The rate of metabolism becomes rapid if serum nicotine level is high. The liver enzyme, CYP2A6 primarily metabolizes nicotine to cotinine, which itself is metabolized by CYP2A6 to 3'-hydroxycotinine and excreted in the urine (29). Nicotine metabolism decreases during fetal life due to immature metabolic enzyme systems (30).

Several studies examined the effects of nicotine on kidneys. However there are few studies that explain the effect of maternal cigarette smoking on fetal kidney (31, 32). Czekaj et al. studied changes in different organs of the fetus exposed to maternal smoking during pregnancy and noticed changes in the epithelial height of proximal convoluted tubules (33). In another study, Gruslin et al. examined the effect of maternal cigarette smoking on fetal erythropoietin concentrations. Increased level of erythropoietin was found in the serum of fetus born to mothers smoking during pregnancies thereby, suggesting that fetal hypoxia was related to maternal cigarette consumption (34). Together, these studies show that exposure to cigarette smoke causes compensatory changes in the kidney however, direct causal link between prenatal smoke exposure and renovascular changes has not yet been provided.

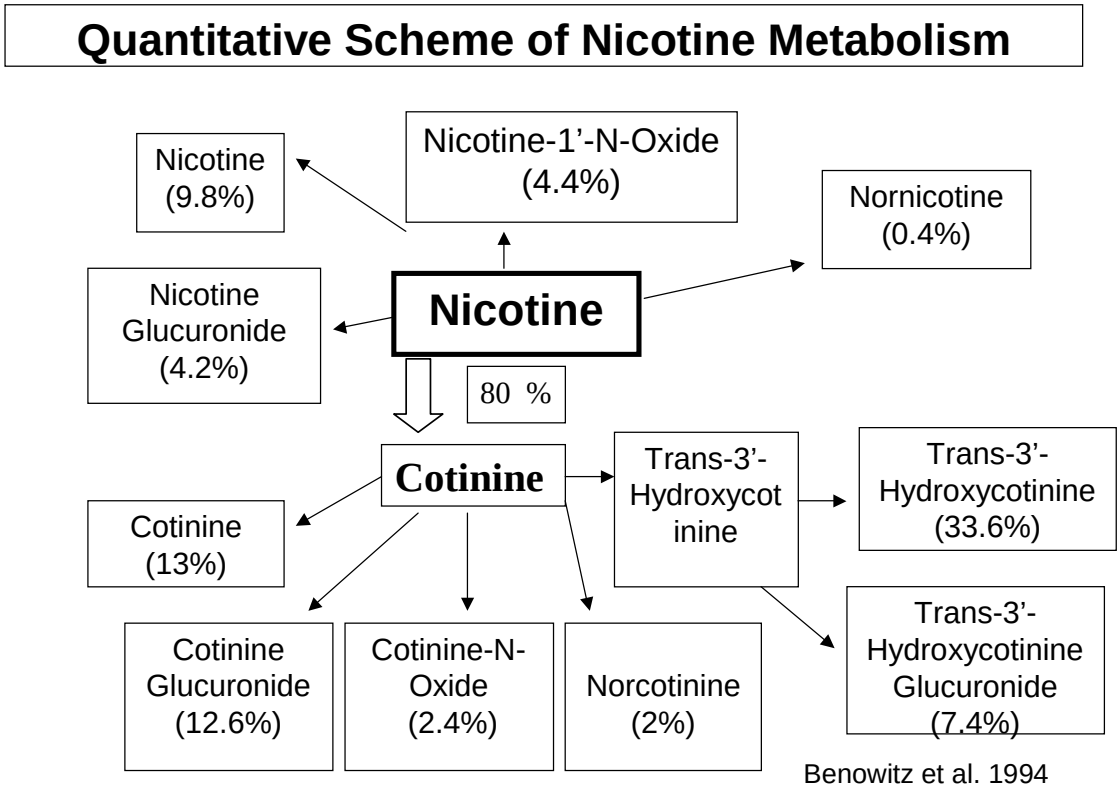

Figure 1.1 Nicotine metabolism flow chart

1.4.2.2 Carbon monoxide

Carbon monoxide (CO) alters the transfer of oxygen through placenta and changes maternal hemoglobin into carboxyhemoglobin and causes maternal and fetal oxidative stress. In various studies, researchers calculated the serum levels of CO in smokers and found that main-stream delivery of CO ranged from 10-20 mg per cigarette and the blood levels of carboxyhemoglobin vary widely from 2 - 14% (mean = 8.1%) in chronic smokers, whereas the reported blood levels in non smoking pregnant women were 0.5-1% and the fetal levels were 0.6 - 1.2%. The mean increase after smoking a cigarette is 0.64% with $t_{1/2}$ of 2 - 4 hours during the day and 8 hours at night (35,36, 37). With the exception of ultra low cigarettes, low and high tar cigarettes yield similar amount of CO (38). Longo et al. studied the effect of CO on fetal oxygen delivery and study results showed an increase in fetal carboxyhemoglobin from 1.8% to 10% and a decrease in the partial pressure of oxygen from 16 to 13 torr, in inferior vena cava (39). In another study, the same authors showed that at level of 30 parts per million (ppm) CO, maternal carboxyhemoglobin increased from 1.1% to 4.6% over 8-10 hours, whereas fetal level of carboxyhemoglobin reached at steady state within 36-48 hours. However, when concentration of CO increased to 100 ppm, fetal and maternal carboxyhemoglobin increased at an exponential rate (40). Recently, researchers in South Korea used breath analysis technique and evaluated the level of carbon mono oxide exposure in active smokers. The results showed that active smoking could cause significant body burden of CO. The post-exposure level of CO in the breath was found to be 1.6 to 2 times

higher than the baseline breath level. It was further observed that on repeated active smoking, CO accumulated in the body (41).

Some researchers have studied the effect of perinatal hypoxia on heart and lungs and observed that even brief perinatal hypoxia causes an increase in the systolic blood pressure of the right ventricle and a decrease in the density of pulmonary artery in rats. On re-exposure to hypoxia, an increase in the severity of pulmonary hypertension was observed (42). Tolson et al. further added that repeated exposure to hypoxia and nicotine could damage cardiac cells (43).

1.4.2.3 Cadmium

Several studies have shown that cigarette smoke could cause an increase in blood levels of Cadmium and may also serve as carcinogen. Shaham et al. compared the blood levels of cadmium in active and passive smokers with unexposed non-smokers and observed that mean cadmium level in the blood was significantly higher in active smokers than unexposed non-smokers while it was very close to the level in passive smokers. It was concluded that blood cadmium level increases by 0.01 micrograms % over the baseline level in active and passive smokers (44). In certain studies cadmium has been found in the amniotic fluid of pregnant women smoking during pregnancy. It was assumed that cadmium might also exert its teratogenic effects on fetus by crossing the placental barrier (45). Studies have also shown that cadmium damages kidney and causes an increase urinary excretion of calcium, amino acids, enzymes and

proteins. Cadmium is also known to increase the level of waste products in blood from different cellular processes (46).

1.5 Maternal Smoking and Low Birth weight

Low birth weight (LBW, <2,500 grams) infants born to mothers, who smoke during pregnancy, are the key predictors for infant mortality. In a recently published study, birth certificates of 79,904 infants born during 1998, in Massachusetts, were reviewed. A total of 11.7% of women acknowledged smoking during pregnancy. An overall rate for LBW was found to be 6.83 and the relative risk (RR) of LBW was 1.58 among smokers. Etiologic fraction for smoking was 6.4% (95% Confidence Interval: 5.4 - 7.3) for all births. The rate of LBW was found to be 6.38% among nonsmokers, 9.5% among light smokers, 11.67% among moderate smokers and 11.72% among heavy smokers on stratification for the effect of smoking (47). In another related study smoking during pregnancy was found to have a profound effect on lowering the birth weight of infants (48).

Ventura et al., in 2003, showed that the incidence of low birth weight among singleton infants who were born to smoking mothers was double as compared to non-smokers. Even light smoking (<5 cigarettes/day) was associated with elevated rates of low birth weight (49). Some researchers then studied the role of other demographic factors on this association between low birth weight and prenatal cigarette smoke. Phung et al. showed that lower fetal birth weight is associated with mothers who are single and of Asian background

and who smoked during pregnancy (50). Zaren et al. observed that cigarette smoke exposure reduced birth weight in male fetus more adversely than female fetus (51). Even studies in developing countries have shown that non-smoking mothers exposed to environmental tobacco smoke have significantly higher incidence of pre-term birth (24.1% vs. 16.1%; p = 0.027) (52).

Smoking during pregnancy has also been shown to be associated with low maternal weight gain during pregnancy. It was shown in a recent study that the odds of low maternal weight gain were 1.34 times greater for smokers than nonsmokers [odds ratio = 1.34, 95% confidence interval = 0.73 - 2.67) (53).

1.6 Maternal smoking and feto-placental changes

Studies have shown that maternal smoking during pregnancy affects fetal growth through changes in the placenta. A recent study showed that degenerative changes and premature aging of placenta caused decreased nutritional supply to fetus and decreased excretion of waste products from feto-placental unit. These placental changes were found to be responsible for fetal growth retardation. Functional changes in placenta also caused hormonal imbalance that could be a cause of premature delivery in smoking mothers (54).

Effects of maternal smoking on fetal circulation were also reported in an epidemiological study. It was concluded that cigarette smoking could change the heart rate of the fetus through its effect on placental circulation (55). In an other

study Genbacev et al. examined adverse effects of maternal first or second hand smoking on placental development and found that maternal smoking adversely affects human placental development by disturbing the regulation of mediators involved in the development of placenta (56).

Certain epidemiological studies have concluded that placental changes resulting from maternal smoking during pregnancy are responsible for low birth weight. Balat et al. in a recent study found significantly lower body weights of newborns and placentas in pregnant smokers (57). Shiverick et al. concluded that the fetus suffered chronic hypoxic stress as a consequence of smoking and a constant diffusive conductance implied for a reduced transplacental partial pressure gradient that could be a contributory factor to the reduced birth weight (58). Demir et al. investigated human placental villi during pregnancy and found that the numbers of syncytial knots and cytotrophoblastic cells increased while mean birth weight and placental weight decreased in the third trimester among smokers in a dose dependent manner (59).

Some researchers have shown that the placentas of cigarette smoking mothers contain an increased cadmium levels, relative volume of maternal intervillous spaces, relative surface area of fetal capillaries, mean thickness of trophoblast component of the villous membrane and a decrease in relative and absolute volume of fetal capillaries. It was concluded that these morphological changes were most likely to be due to compromised transplacental oxygen

transfer (60). In 2003, same authors examined the hemodynamic changes in maternal circulation. They showed fibrinoid deposits in placental intervillous spaces causing coagulation disorder that could affect transfer of nutrients from mother to fetus (61).

Pastrakuljic et al. showed the association of cocaine and cigarette smoking in pregnancy with the impaired transplacental amino acid transport that might be responsible for intra uterine fetal growth restriction (62). Habek et al. assessed the effect of smoking and its metabolites on feto-placental unit. Chronic hypoxemia due to carboxyhemoglobinaemia, chorionic and placental impairment, vasoconstriction of utero-placental circulation and intermediary metabolic disturbance was considered to be an etiologic basis of intrauterine fetal growth retardation (63).

Godfrey et al. examined the effects of high dietary intake on placental growth. They observed that the women with high dietary in take in early pregnancy have smaller placentas. The babies with a disproportionately smaller placenta had an impaired placental supply of nutrients while those with disproportionately large placentas might have fetal catabolism and wasting to supply amino acid for placental consumption. This resulted in fetal adaptation that increased adult coronary heart death rate in those with both low and high Placental Ratio, the ratio of placental weight to fetal birth weight (64).

1.7 Maternal smoking, low birth weight and hypertension

Epidemiological studies have revealed an inverse relationship between birth weight and blood pressure in the offspring of smoking mothers as compared to non-smokers. The underlying factors proposed by Lackland et al. for this relationship included reduced nephrogenesis, greater susceptibility to progressive renal disease, impaired development of endothelium, and increased sensitivity to glucocorticoids (65).

Data collected by Szathmari et al. suggested that in addition to the intrauterine retardation the sudden break of fetal development and an accelerated pace of early postnatal growth might have played a role in the pathophysiology of chronic diseases like hypertension (66). However, Blake et al. concluded that intra-uterine exposure to maternal cigarette smoking increases children's blood pressure at age one through six years. This was not solely attributable to an effect on birth weight or confounding through association between birth weight and subsequent blood pressure by the child's current weight or socio-economic factors (67).

Steuerer et al. proposed that oxidative stress is a contributing factor in low birth and adulthood disease like hypertension. It was shown that children of smoking mothers with a low birth weight have significantly lower vitamin E concentrations. Smoking during pregnancy increases the consumption of vitamin E, so that there is an overproduction of peroxides and a reduction in prostacyclin.

This lack of prostacyclin led to diminished perfusion of the placenta resulting in low birth weight infants (68). Franco et al. on the other hand showed that intrauterine under nutrition enhanced oxidative stress *in vivo*. This increased oxidative stress provided a potential explanation for the endothelial dysfunctional development resulting in hypertension in adulthood (69).

1.8 Fetal programming and adulthood disease

Perinatal programming or 'fetal origin of adult disease' is a concept that arose after the publication of epidemiologic observations by Barker and colleagues. They proposed that birth weight among apparently normal individuals studied in midlife was inversely associated with the presence of cardiovascular disease and hypertension (70,71). The concept of fetal programming then opened the field for extensive research into the fetal origin of adult diseases (72).

Moritz et al. demonstrated that genetic and life style factors have a programming effect of an adverse fetal environment. Kidney was suggested to be the major organ affected by intrauterine environment. The factors that can affect fetal renal development also produce hypertension in the adult animal. It was observed that certain maladaptive functional changes leading to hypertension occurred when nephron number compromised during kidney development (73).

Woods et al. suggested that intrauterine environment is an important factor that determines the future health of the baby. Factors involving intrauterine

environment can cause permanent changes in the structure and function of specific tissues in the body. They observed that the renin-angiotensin system was central in setting the trajectory that led to cardiovascular disease particularly hypertension (74).

Marchand et al. showed that maternal nutritional status in pregnancy was a major programming influence upon the fetus. They considered the hypothesis that nephron number in humans was determined by prenatal nutrition (75). A rat model of intrauterine growth retardation, induced by maternal low-protein diet during the second half of pregnancy, was used by Manning et al. to study the relationship between birth weight and adult hypertension. Both Sprague-Dawley and Wistar rat strains developed equally severe hypertension after maternal protein deprivation, despite their different susceptibilities to nephrosclerosis with aging. Primary sodium retention and expanded extracellular volume were found critical factors during the development of hypertension (76). Vehaskari et al. observed that the kidney and body sizes of the offspring from the low-protein pregnant Sprague-Dawley rats were proportionately decreased at birth. The total number of glomeruli per kidney was decreased by 28% in males and by 29% in females. By the age of eight weeks, both male and female low-protein pregnant rats had systolic blood pressures that were 20 to 25 mm Hg higher than those of control animals (77).

Merlet-Benichou et al. in their two studies concluded that changes in the

supply of vitamin A to the fetus might be responsible for the variations in the number of nephron in the human kidney (78,79). Battista et al. provide evidence that a low-sodium diet, given to pregnant rats over the last week of gestation, resulted in intrauterine growth restriction. It was further proposed that pups born with intrauterine growth restriction were susceptible to the development of hypertension in adulthood. Brain and cardiac ventricle-to-body ratios were increased at 12 weeks in intrauterine growth restriction fetuses compared with age-matched controls, whereas kidney-to-body ratio was unchanged. Systolic blood pressure was elevated in both intrauterine growth restricted male and female adults (80).

Woods et al. in their experimental study fed a normal protein (19%) or low-protein (8.5%) diet to pregnant rats throughout gestation. Birth weight was reduced by 13% and the kidney/body weight ratio was reduced in low-protein pups. Renal renin mRNA level was significantly reduced and renal renin concentration and renin immunostaining were suppressed in newborn low-protein pups. Renal tissue angiotensin II level was also suppressed in newborn low-protein pups. Mean arterial pressure in adult offspring was higher in the low-protein group. Glomerular filtration rate and number of glomeruli per kidney was reduced in low-protein offspring. It was proposed that prenatal protein restriction in the rats suppressed the newborn intrarenal renin-angiotensin system and led to reduced number of glomeruli, glomerular enlargement, and hypertension in the adult (81).

Fogo et al. suggested that the renin-angiotensin system could be linked to induction of plasminogen activator inhibitor-1, possibly via the angiotensin IV receptor, thus promoting both thrombosis and fibrosis. Interactions of the renin-angiotensin system with aldosterone and bradykinin may have impact on both blood pressure and tissue injury (82). Matsusaka et al. observed that kidney develops with profound structural abnormalities when angiotensin II or I receptor is experimentally inhibited during the prenatal period. Mutant mice completely devoid of angiotensin I receptors fail to develop peristaltic movement in the renal pelvis and the ureter (83).

Langley et al. performed experiments with prenatal under nutrition in rats. It was clear that fetal exposure to glucocorticoids of maternal origin was the first step in the programming of hypertension and coronary heart disease. The chain of events leading from glucocorticoid action in the fetal tissues to hypertension in adulthood involved the development of hypersensitivity to glucocorticoids in adult life. This had the effect of activating the renin-angiotensin system through induction of key genes such as ACE, which increased the sensitivity of the blood vessels to the actions of angiotensin II (84). Zoccali et al. also observed that subjects homozygous for the D allele of the ACE gene were predisposed to both cardiovascular complications and nephrosclerosis, suggesting that genetic factors might interact with altered intrauterine growth in determining the risk of cardiovascular and renal diseases (85).

Experimental data in animals and recent human observations suggested that an alteration in the set point of the hypothalamic-pituitary-adrenal axis was an important long-term change that occurred in association with reduced fetal growth. Such observations raised the possibility that the nature and amplitude of the stress response might be determined by intra-uterine factors (86). More recently, it has been shown that an important determinant of adulthood hypertension was the early exposure of the developing fetus to excess of glucocorticoids. Hypertension developed in adult sheep and rats that are exposed to excess of glucocorticoids at a gestational stage when both kidney and brain are still extremely primitive organs (87). Dodic et al. proposed that permanent changes in gene expression and function of these two organs could be crucial in the development of adult-onset hypertension as a result of prenatal glucocorticoids exposure (88).

Araujo et al. observed the effect of tonins that are mainly found in the submandibular gland of rat. These are capable of generating the pressor octapeptide angiotensin-II not only from the classical substrate angiotensin -I but also from the synthetic tetradecapeptide and from angiotensinogen. The results showed that central microinjection of tonin produced a transient (10-20 min) elevation of blood pressure and heart rate and induced water and saline intake within the first 10 min after injection. Urinary volume and salt excretion increased within 7 hours after injection. These effects were partially blocked by previously administered losartan (angiotensin II receptor antagonist), indicating that tonin

effectively induced a central angiotensin II formation. The authors suggested that tonin might be an alternative pathway to angiotensin-II generation in the brain and could participate in the physiological effects exerted by angiotensin-II such as water and saline intake and blood pressure elevation (89).

Wintour et al. has recently proposed that all the stresses to which mother is exposed during pregnancy are associated with 20-40% reduction in total nephron number in the adult, and the development of hypertension. In some hypertensive models in rats, there was evidence of alterations in the components of the hippocampal-hypothalamic-pituitary-adrenal axis, whereas in others models in sheep, there were alterations in the expression of angiotensinogen (hypothalamus) and angiotensin-II receptor type I in the medulla oblongata. The surprising finding was that the period i.e., kidney and brain were most vulnerable in an extremely primitive state of development (90).

1.9 Fetal Programming and Cardiovascular Changes

In an epidemiologic study, Zhang et al. showed that factors like maternal cigarette smoking, alcohol intake and under nutrition caused fetal hypoxia. It was observed that these factors could result in decreased fetal cardiac functions, increased apoptosis of myocytes and decreased number of cardiac myocytes. These changes consequently caused compensatory hypertrophy of cardiac myocytes. They concluded that intrauterine environment has an impact on the development of hypertension and coronary heart disease in adulthood by

causing fetal growth retardation (91). In a study performed on an animal model, Longo et al. observed the adverse effect of intrauterine environment on fetal vascular function in adulthood and proved vascular dysfunction in carotid and mesenteric arteries (92).

In another epidemiological study, changes in cardiovascular function were observed during a stress challenge test. It was concluded that there was a relationship between low birth weight and adulthood cardiovascular response under stressful conditions (93). Amann et al. in 2004, provided evidence that individuals with low birth weight had lower number of nephrons in their kidneys. In this study, renal dysplasia was also found thus suggesting that these prenatal renal changes could be a cause of cardiovascular diseases in adulthood (94).

1.10 Fetal Programming of Hypertension and Reno-vascular Changes

It was suggest by some researchers that fetal exposure to maternally derived glucocorticoids plays a key role in the programming of hypertension. The results showed that the offspring of rats fed on low-protein diet exhibited a number of metabolic and physiological disturbances, and consistently found to have high blood pressure from early postnatal life. It was concluded that secondary to this activity, fetal hypothalamic-pituitary-adrenal axis stimulated renin-angiotensin system activity, resulting in increased vascular resistance and hypertension (95). Ingelfinger et al. also observed the effects of maternal nutrition and health on fetal nephrogenesis and found that renal function changes due to

tubular changes in later life, when nutritional alterations compromised during pregnancy at the time of nephrogenesis (96).

Lamireau et al. showed that reduced endothelium-dependent vasorelaxation, partly due to loss of nitric oxide bioavailability, occurred in most of the cases of chronic hypertension. Intrauterine nutritional deprivation was found to be associated with increased risk of hypertension and stroke, and decreased vascular compliance. However, underlying mechanisms remained unknown (97). Some other researchers were able to provide explanation for their results. Franco et al. showed that intrauterine under nutrition enhanced oxidative stress *in vivo* and related this stress to impaired endothelium-dependent vasodilatation in hypertensive offspring (98). Hanson et al. suggested that reduced vasodilatation might be a potential mechanism underlying the elevated systolic blood pressure in offspring of low protein fed rats, during pregnancy (99).

The above overview of previous studies provides evidence that maternal nutritional status or adverse intrauterine environment have a programming influence on fetus for adulthood diseases like hypertension and reduced number of nephrons and kidney size. However, current literature provides limited information on prenatal cigarette smoke exposure causing histological changes in kidney that may alter blood pressure at adulthood. The present study is an effort to explore these associations.

Chapter two: Materials and Methods

Chapter two provides information on the experimental design, operational definitions, sampling techniques, data collection procedure and study variables along with data analysis. It also provides detailed information on smoke exposure and telemetry systems that were used in the current study. Stereo-investigator system and microtomy procedure have also been explained. An account of ethical considerations taken at each step of the study has also been provided.

2.1 Study design

In the current study, age matched rats of various ages were utilized as a model of cigarette smoke exposure. The study was performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the Animal Care Committee of the University of Calgary.

2.1.1 Selection of animals

Wistar Kyoto rats were obtained from Charles River Laboratories, Inc. The rational for using Wistar Kyoto strain is as follows (100).

- This strain is readily available.
- The rats are normotensive and have been used extensively in cardiovascular research.

- The results were deemed easier to compare with other international publications using this model.
- All the previous studies in our lab have been performed on Wistar Kyoto strain of rats.
- Larger size of the rat made implantation of the radio-telemetry probes easier.
- It is the most commonly used stain for postnatal and fetal developmental studies (101).

2.1.2 Animal breeding

Rats were kept in the Animal Resource Center, University of Calgary. The room temperature was kept at 20^0 Celsius and 12-hour light and dark cycle was maintained throughout the experiment. After acclimatization, the animals were divided in to two groups; smoke and sham. Rats in both groups were placed into their breeding cages. One pair of male and female was place in one breeding cage. Pregnancy was confirmed by the presence of vaginal mucous plugs in the breeding cages.

2.1.3 Study groups

Nose-only Smoke Exposure System was used and eight pregnant female rats from the smoke group were exposed to cigarette smoke for the entire duration of gestation, which is equivalent to 23 days. The rats were exposed to cigarette smoke equivalent to seven cigarettes per day throughout pregnancy.

There were ten puffs in one cigarette and one puff produced 15 ml of smoke. The total volume (dose) of cigarette smoke was calculated by multiplying puff volume with number of puffs and cigarettes per day. As a whole, rats were exposed to 1050 ml cigarette smoke daily.

For the comparison purpose, eight pregnant rats were taken as sham group and were exposed to air through smoke exposure system. They were subjected to the same handling procedure and environment as that of smoke exposed group and had similar free access to food and water like rats in the smoke exposed group.

For the breeding purpose, each group had eight male and eight female rats. Rats in both groups (cigarette smoke and sham group) were kept in breeding cages as described above. One cage had one pair, male and female. Eight pregnant female rats from each group were either exposed to smoke or air depending upon their group assignment. After delivery, offspring were kept with the mother in both groups. On postnatal day 16, one offspring from each liter in both groups (total of 8 offspring per group) was randomly selected and sacrificed for the removal of kidneys. On postnatal day 120, one offspring from each liter in both the groups (total of 8 offspring per group) was randomly selected and telemetry system was implanted in the abdominal cavity and a probe was placed in the femoral artery to record blood pressure and heart rate. On postnatal day

200, the abdominal implant and probe were removed and all offspring were sacrificed to remove kidneys.

2.2 Smoke exposure system

The nose-only Smoke Exposure System was developed at University of Kentucky. This system was used to expose rats in the smoke group, to cigarette smoke and rats in the sham group to air (102). It provides mainstream cigarette smoke to up to eight rats from the same cigarette.

2.2.1 Components of smoke exposure system

Smoke exposure system had three main units

- Smoke generation
- Smoke distribution
- Animal exposure

Figure 2.1 provides details of the nose-only smoke exposure system.

2.2.1.1 Smoke Generation

This unit consisted of a puffer, pump and sidestream smoke collection chamber. The puff volume could be adjusted to any desired amount by adjusting the Cam on the puffer or by calibration of air/smoke flow through the main vacuum line, waste line and pump’s head line. One puff of smoke per cigarette was produced every minute and lasted approximately 2.4 seconds. The smoke from each puff then flowed to the smoke distribution units.

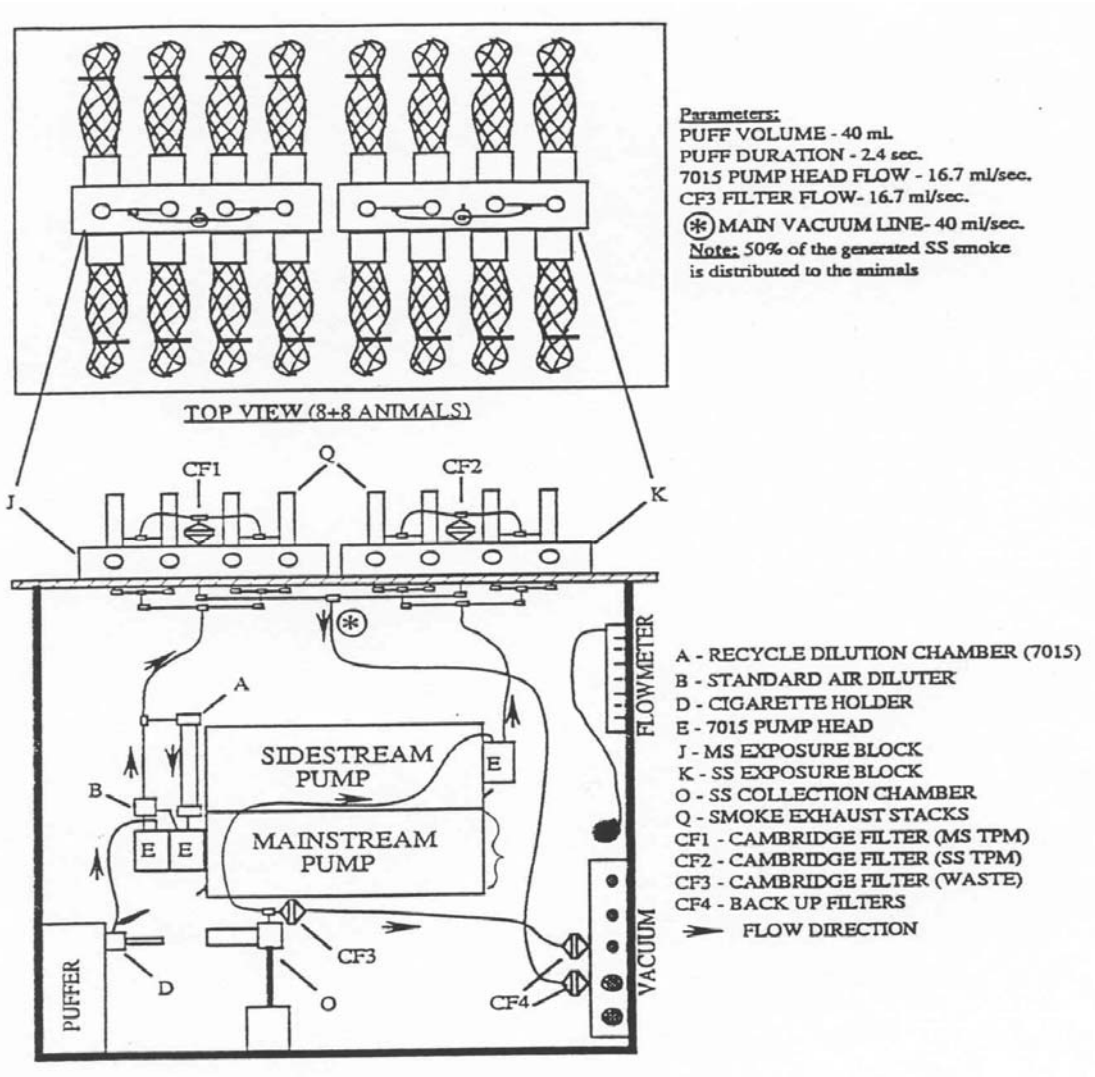

Figure 2.1 Nose-only Smoke Exposure System

2.2.1.2 Smoke distribution unit

The mainstream distribution unit consisted of one pump, two pump heads (7015), and a recycle dilutor (chamber). Both mainstream and side stream smokes were distributed to the exposure blocks. In this study, we used mainstream cigarette smoke. The mainstream cigarette smoke was equally distributed to all eight rats and was diluted to 50% before delivery. The smoke took about 15-30 seconds to clear the recycle dilutor.

2.2.1.3 Smoke exposure

The smoke exposure portion had two blocks, main steam smoke and side stream smoke. It was set up to expose eight rats to mainstream and /or eight rats to side stream smoke simultaneously from the same cigarette. Unused exposure parts were closed using rubber stoppers.

2.2.1.4 Additional parts

Additional parts of the system included a vacuum source, a flow meter used for airflow calibrations, and CF1, CF2, and CF3 filters, which were placed in the given locations and were changed three times daily.

2.2.2 Operation and Maintenance

The mainstream pump was warmed up for at least 10 minutes. Calibration of smoke exposure system was carried out every day after the last cigarette and a control run (without rat) was performed to monitor the machine’s performance.

In addition, pump head tubing was changed daily and other machine's parts were cleaned with 70% ethanol. Before the exposure, rats were placed in wire mesh to restrain and to be loaded into the machine. The sham group was exposed to air only. For this procedure, we replaced the mainstream pump by a new pump maintained at the same speed.

2.3 Research Cigarette

Research cigarette (1R3) was obtained from the Tobacco and Health Research Institute, University of Kentucky. A description of the cigarette included, a draw resistance of 7.4 cm of H_2O, 550 static burn sec/40 mm, 85 mm length, 25.1 mm circumference, paper porosity of 47.6 sec/50 cc and nicotine capacity of 2.16 mg. The cigarette was stored at 3.3 Celsius and 50-60% humidity at Tobacco and Health Research Institute. Prior to shipment, the cigarette package was removed from cold storage and placed in a temperature and humidity controlled room at 23.8 Celsius and 60% relative humidity for equilibration. After arrival in our laboratory, the cigarette was stored in a standard laboratory refrigerator until it was used in the experiment. This procedure was adopted in accordance with the recommendation of the Tobacco and Health Research Institute to maintain moisture level in the cigarettes.

The rats were exposed to smoke from seven cigarettes daily up to twenty-three days of pregnancy. There were ten puffs in one cigarette and one puff produced 15 ml of smoke. It means that the total daily dose of cigarette smoke

was 1050 ml (7 cigarettes x 10 puffs x 15 ml volume). It was based on our previous work that 1050 ml of cigarette smoke per day causes plasma concentration of nicotine equal to 65 ng/ml in pregnant rats. Weight of each pregnant rat in both the groups was recorded every day.

2.4 Telemetry System

The telemetry system used in this study was designed by “Data Sciences International” to provide continuous recording of heart rate and blood pressure without subjecting an animal to strain or stress (103). This system could work up to 6 months on a number of different animal species.

2.4.1 Components of the telemetry system

There were five main components of the telemetry system.

- Implant
- Receiver
- Data exchange matrix
- Data acquisition system and
- Ambient pressure reference

An explanation of each component is given in the following lines. Details have also been provided in Figure 2.2.

2.4.1.1 Implant

It consisted of a small capsule like structure with a 10 cm catheter attached at one end. The tip of the catheter contained a sensor for blood pressure measurement. The complete implant weighing approximately 9 grams, was implanted in the abdomen of rat. The catheter was inserted in the right femoral artery. The implant sent a radio signal carrying the measurements to the receiver.

2.4.1.2 Receiver

The receiver was a flat square panel that was placed under the cage of each rat carrying implant. It received radio signals from the implant and forwarded them to the data exchange matrix.

2.4.1.3 Data Exchange Matrix

This component detected the type and serial number of the receiver and passed on collected data to the acquisition system. In the current study, one data exchange matrix was used to receive information from 16 receivers (as there were 16 rats in the study).

2.4.1.4 Data Acquisition System

It consisted of software that could collect and store data. This system was set to record continuous sampling and provided calculated mean values.

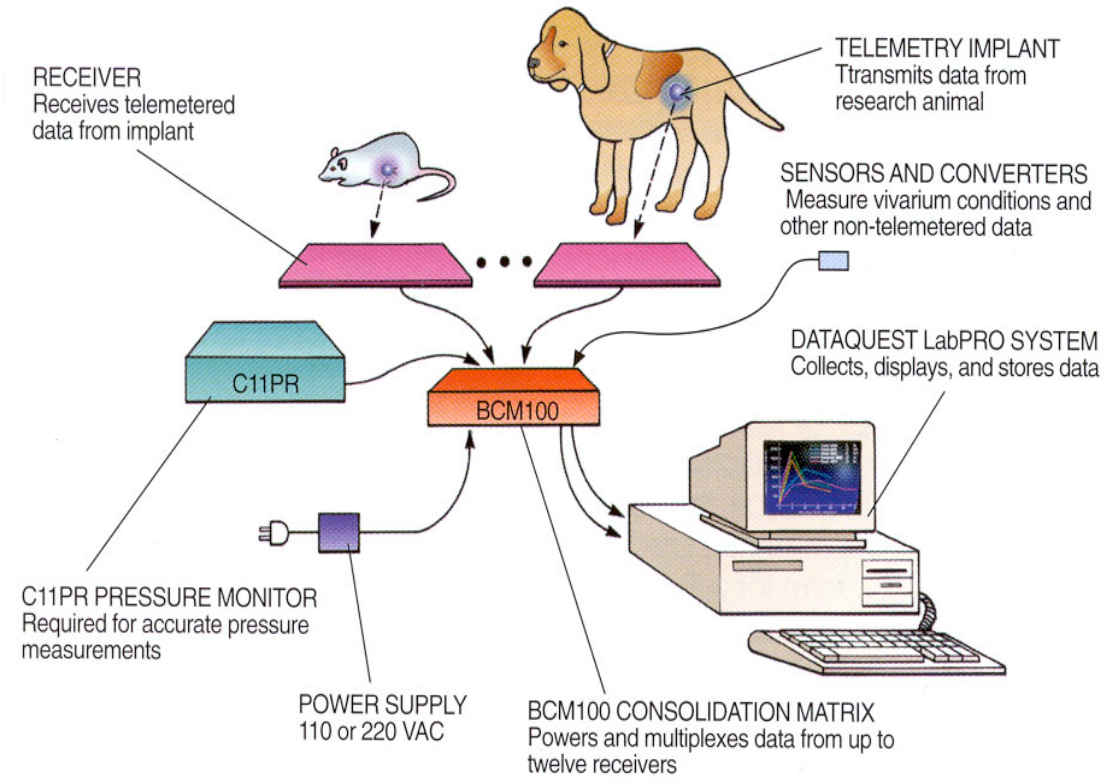

Figure 2.2 Components of the telemetry system

After collection, data was passed to another computer program especially designed by Data Science Inc., for further analysis.

2.4.1.5 Ambient Pressure Reference

The ambient pressure reference was maintained to keep a record of room barometric pressure. It facilitated accurate measurement of blood pressure by recording any change in the ambient pressure and transmitting that information to a computerized system. The acquired values were then automatically corrected for any change in barometric pressure.

2.4.2 Operation of telemetry system

One could set this system for either continuous or scheduled sampling. In the present experiment, we maintained continuous sampling. Recording of the variables was taken for ten seconds every five-minute for 24 hours. Implants was turned on for taking measurements and turned off after 24 hours to preserve battery of the implant. A radio was used to check the working conditions of the implant and to maintained appropriate distance between two receivers.

2.4.3 Surgical procedure to implant telemetry device

The telemetry implant (device) was surgically implanted in each rat. Implant battery was turned on two days prior to surgery and its offset value was checked to confirm the working condition of the probe. For cleaning purpose, the implant was initially soaked in an enzymatic detergent, Terg-a-Zyme. It was then

soaked for 12 hours in a sterilizing solution, Cidex. The implant was rinsed with normal saline solution before placing it inside the abdominal cavity of rat. Each rat was weighted prior to surgery. Surgery was preformed under general anesthesia using 1% Halothane in 100% oxygen mixture. The abdominal surface and right thigh were shaved and sterilized using iodine and alcohol. Abdominal cavity was opened by 1.5 cm long vertical incision in the midline. The second incision (1 cm) was given on left thigh parallel to the femoral artery (Figure 2.3). The implant was placed in the abdominal cavity and catheter was pierced through abdominal wall in to the femoral area. The femoral artery was exposed and dilated by 1-2 drops of 2% xylocaine solution. A catheter was inserted into femoral artery with the help of a 22½ gage needle bent at 90º angle. The needle was removed and catheter was pushed upwards inside the artery. A silk ligature was tide firmly around the artery and catheter to avoid it from slipping out of the artery. One ml sterile solution was infused in the abdominal cavity in order to replace fluid lost during surgery. The body of the implant was sutured to the abdominal wall through three openings in the capsule of the implant. Both, abdominal and thigh incision were sutured using 4-0 absorbable vicryl. Butorphanol tartrate, 2 mg per kilogram body weight, was given subcutaneously immediately after surgery to prevent post surgical pain.

The entire surgical procedure was performed under sterilized conditions. Rats were placed in separate clean cages after surgery. All operated rats were

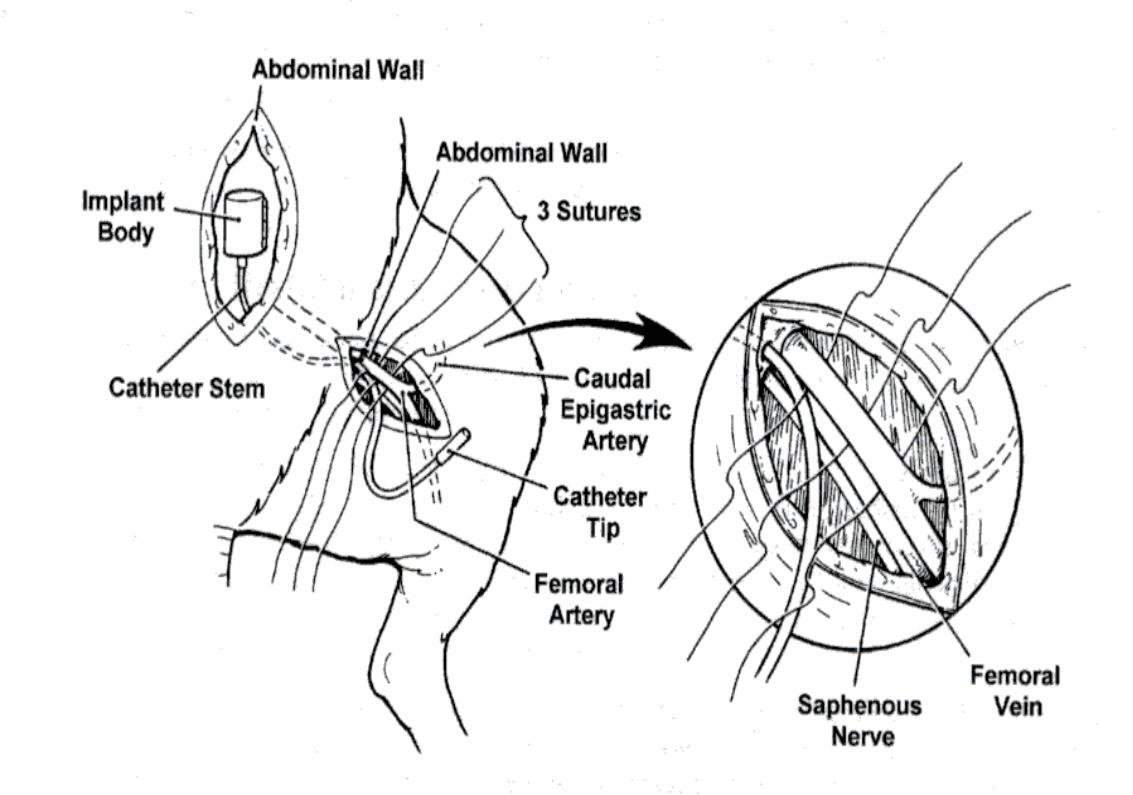

Figure 2.3 Surgical procedures for implantation of telemetry system

kept under observation in an isolated room for anesthesia recovery. An oral dose of acetaminophen mixed in drinking water was given for three postoperative days to relieve discomfort. The rats were checked daily for wound healing, and weighed for ten days in order to assess their post-surgical health.

2.4.4 Telemetry protocol

The telemetry system was organized in two rooms, which were situated inside the Animal Resource Center. The computer was placed in one room while receivers, cages and ambient pressure system were placed in another room. Cords and electric wires were passed through an opening in the door between the two rooms to connect various pieces of equipment. Access to the laboratory was restricted during the recording hours.

Dataquest telemetry software program was installed in a specific computer to record time, systolic blood pressure, diastolic blood pressure, mean blood pressure, pulse pressure, heart rate, and activity of rats. The recording was started on postnatal days 160 and continued up to postnatal day 200. To warm up the equipment, the implant was turned on 24 hours before taking the measurements. Each measurement was recorded for ten seconds after every five minutes for 24 hours. The recordings were made for each rat in both groups. On completion of recordings, data was transferred to “Microsoft Excel” for further analysis.

2.5 Morphometry

2.5.1 Removal of kidneys

On postnatal days 16 and 200, rats were sacrificed using a lethal dose of pentobarbital (40-60 mg/kg-body weight). A 23-gauge needle was used for intraperitoneal injection of pentobarbital (96). Abdominal cavity was opened and right kidney of each rat was dissected and removed. The kidneys were weighed and fixed in 10% buffered formaline. Each kidney was kept in a separate container and labeled.

2.5.2 Procedure for Microtomy

The organs were kept for 2 days in formaline solution for fixation. Longitudinal midline sections of each kidney were made. The sectioned kidneys were placed in labeled plastic cassettes and submerged and saved in 10% formaline solution.

Research area of histopathological laboratory at Foothills Medical Center, was used for microtomy and staining procedures. The following steps were taken for manual microtomy (104).

- The sectioned kidneys were submerged in paraffin and blocks were prepared.
- Excess wax from all sides of the paraffin blocks was trimmed.

- Blocks were placed in the holder of the microtome by pulling on the release clamp and inserting blocks.
- Blocks were marked subsequently at the top.
- To expose the rough surface of the tissue the blocks were trimmed again and set in numerical order to cool on ice tray.
- Cooled blocks were placed in a holder of the microtome by pulling on the release clamp and inserting blocks
- Micrometer dial was set and blocks were aligned to a knife-edge and trimmed again until the surface was re-exposed
- Rotary wheel was turned until a ribbon was produced for each block and each ribbon was floated on water that was kept at temperature 42 – 48 Celsius
- Each ribbon was centered on a labeled glass slide and drained
- Each slide was placed on a hot plate at temperature 58-62 Celsius for 5 - 7 minutes to melt wax

2.5.3 Staining of slides

Following steps were taken for staining the slides (105).

- Staining basket was held and starting at the rear, Hematoxylin and Eosin (H&E) control slide and then slide with the surgical number was put in.
- Stainer and water was turned on
- Hematoxylin was put on the stainer and “start” button was pushed

- H&E method was selected and pushed “select” button followed by “start” button
- Second basket was loaded by pressing “add” button
- Pushed “start” button. There was alarm when staining of the slides was finished.
- Pressed “exit” button and removed the basket
- Pressed “confirm” button and slides were checked by to ensure that the slides were acceptable.

2.5.4 Stereology

Stereology is a mathematical measurement of a three-dimensional object by making two-dimensional section. It can help to estimate geometrical quantities like surface area, numbers and volumes (106).

The prepared slides were examined under light microscope. A digital camera was attached to the light microscope, which was in turn connected to a computer containing Stereo-Investigator Program. Each slide was examined with the help of “fraction- fractionators area” probe. Information on following variables was collected through this system:

- Cortical fraction area
- Glomerular fraction area
- Ratio between glomerular and cortical fraction areas

2.6 Operational definitions

For accurate measurement, all the variables were provided with operational definitions.

Smoke exposed group

Offspring of the rats exposed to cigarette smoke using smoke exposure system during pregnancy.

Sham group

Offspring of the rats exposed to puffs of room air using the smoke exposure system during pregnancy.

Body weight

Body weight, in grams, of each rat in both the groups, on postnatal days 16 and 200

Kidney weight

Weight (grams) of right kidney of each rat in both the groups, on postnatal days 16 and 200

Glomerular fraction area

The area (micro-meter square) of glomeruli present in one slice of each kidney, as observed in a slide on stereology.

Cortical fraction area

It is the area (micro-meter square) of cortex present in one slice of each kidney, as observed in a slide on stereology.

Ratio between glomerular and cortical fraction areas

It is the ratio between glomerular fraction area and cortical fraction area

Systolic Blood Pressure

Blood pressure, in mm of Hg, recorded through telemetry system during the contraction phase of heart, on postnatal days 160 and 200 for each rat. Systolic blood pressure more than or equal to 130 mm Hg was considered hypertension.

Diastolic Blood Pressure

Blood pressure, in mm of Hg, recorded through telemetry system during relaxing phase of heart, on postnatal days 160 and 200 for each rat. Diastolic blood pressure more than or equal to 90 mm Hg was considered hypertension.

Mean Blood Pressure

Average of systolic and diastolic blood pressures as recorded by the sensor of the probe placed inside femoral artery.

Pulse Pressure

Difference between systolic and diastolic blood pressures as recorded by the sensor of the probe placed inside femoral artery.

Heart Rate

Normal heart rate of Wister Kyoto rats varies from 296 to 388 heartbeats per minute. Heart rate for this particular study meant the heartbeats recorded in one minute, through telemetry system, on postnatal days 160 and 200 for each rat.

2.7 Variables

Keeping in view the objectives of the study, data was collected for following variables for both the groups.

Variables recorded for the primary objective

- Body weight (grams)
- Kidney weight (grams)
- Glomerular fraction area (micrometer square)
- Cortical fraction area (micrometer square)
- Ratio between glomerular and cortical fraction areas

Variables recorded for the secondary objective

- Systolic Blood Pressure (mm Hg)
- Diastolic Blood Pressure (mm Hg)
- Mean Blood Pressure (mm Hg)
- Pulse pressure (mm Hg)
- Heart rate (beats/minute)
- Diurnal variation in blood pressure
- Percent change in diurnal variation of blood pressure

2.7.1 Body weight

Body weight of each offspring in both groups was recorded on postnatal

days 16 and 200 as an indicator of change in postnatal growth and to compare any change in body weight with variation in kidney weight.

2.7.2 Kidney weight

In order to keep uniformity and fair comparison between smoke and sham groups, only right kidney of all offspring was weighed on postnatal days 16 and 200.

2.7.3 Glomerular fraction area

One offspring per liter was randomly selected from the smoke and sham groups and glomerular fractional area per cortex of each kidney was calculated through stereology. Information was recorded on postnatal days 16 and 200.

2.7.4 Cortical fraction area

One offspring per liter was randomly selected from both the groups and cortical fraction area per section per kidney was calculated through stereology and information was recorded on postnatal days 16 and 200.

2.7.5 Ratio between glomerular and cortical fraction areas

This was the main outcome variable for the first objective of the current study. Ratio between glomerular and cortical fraction areas per kidney was calculated on postnatal days 16 and 200. Information was recorded for each offspring in both the groups.

2.7.6 Cardiovascular variables

It included systolic blood pressure, diastolic blood pressure, mean blood pressure and pulse pressure, and heart rate. Information on these variables was recorded through telemetry system, on postnatal days 160 and 200.

As rat is a nocturnal animal, information on diurnal variation in diastolic, systolic and mean blood pressure was also recorded through telemetry system on postnatal days 160 and 200.

2.8 Data analysis procedure

2.8.1 Descriptive statistics

Each variable has been described in terms of mean and standard deviation (SD). A comparison between the smoke and sham groups, on each variable, has been displayed in a tabular form along with description of salient features.

Diurnal variation in blood pressure has been presented graphically and comparison between both the groups have been made in the morning and evening hours. Comparison has been further elaborated through graphic presentation of percent change in diurnal variation in blood pressure. Slides providing information on histopathological changes in kidneys of both the groups has also been given in the result section.

2.8.2 Analytical statistics

Observed difference between the two groups, on each variable, across time was analyzed using ANOVA for repeated measures (Manova). However, average tests were identical to the univariate tests of significance. The null hypothesis was rejected if p-value was less than or equal to 0.05. Each test result providing values of “F” and “p” has been displayed. An interpretation of the results on each variable has been given. However, using *t* test for independent variables made comparison between the two groups on percent change in diurnal variation of blood pressure. The test results for all the comparisons have been displayed in the form of a table. A paragraph interpreting p-value and 95% confidence interval followed the results.

Chapter Three: Results

3.1 Results related to primary objective

The primary objective of the current study was to investigate histological changes in the kidney of male rat at adulthood secondary to prenatal cigarette smoke exposure. However, data was also collected on other relevant variables as described in the previous section. Results related to this objective are given below.

3.1.1 Body weight

3.1.1.1 Body weight on postnatal day 16

Numeric comparison, on this variable, between the two groups is provided in Table 3.1.

Table 3.1 Numeric comparison of body weight between the smoke and sham groups, on postnatal day 16

Group	Number of offspring	Mean (grams)	SD
Smoke	8	23.50	2.86
Sham	8	29.18	1.85

As shown in Table 3.1, mean body weight of offspring in the smoke group was less than that of the sham group, on postnatal day 16.

Manova was performed to evaluate the observed difference statistically. The test result was significant ($F_{1, 14} = 22.21$, $p < 0.001$). Therefore, mean body weight of offspring in both the groups was significantly different on postnatal day 16. It means prenatal exposure to smoke significantly reduced the mean body weight of offspring.

3.1.1.2 Body weight on postnatal day 200

Table 3.2 provides numeric comparison on this variable between the two groups.

Table 3.2 Numeric comparison of body weight between the smoke and sham groups, on postnatal day 200

Group	Number of offspring	Mean (grams)	SD
Smoke	8	383.89	23.09
Sham	8	384.25	14.94

As shown in Table 3.2, mean body weights of offspring were similar in both groups, on postnatal day 200.

The result of Manova was insignificant ($F_{1, 14} = 0$, $p = 1$). Therefore, mean body weight of offspring, in the two groups, was not significantly different on postnatal day 200. It means, with the passage of time, offspring overcame reduction in body weight caused by prenatal cigarette smoke exposure.

3.1.2 Kidney weight

3.1.2.1 Kidney weight on postnatal day 16

Table 3.3 provides a summary of numeric comparison between kidney weights the smoke and sham groups.

Table 3.3 Numeric comparison of kidney weight between the smoke and sham groups, on postnatal day 16

Group	Number of offspring	Mean (grams)	SD
Smoke	8	0.134	0.014
Sham	8	0.186	0.042

As shown in Table 3.3, offspring exposed to prenatal cigarette smoke had lower mean kidney weight as compared to the sham group, on postnatal day 16.

Manova was performed and result was significant ($F_{1, 14} = 11$, $p = 0.005$). Therefore, mean kidney weight of offspring in the two groups was significantly

different on postnatal day 16. It means prenatal exposure to smoke significantly reduced the kidney weights of offspring.

3.1.2.2 Kidney weight on postnatal day 200

Numeric comparison between the smoke and sham groups is provided in Table 3.4.

Table 3.4 Numeric comparison of kidney weight between the smoke and sham groups, on postnatal day 200

Group	Number of offspring	Mean (grams)	SD
Smoke	8	1.213	0.146
Sham	8	1.325	0.205

As shown in Table 3.4, mean kidney weight of offspring was similar in both the groups on postnatal day 200.

Manova was performed. The test result was insignificant ($F_{1, 14} = 1.6$, $p = 0.23$). Therefore, mean kidney weight of offspring in both the groups was not significantly different on postnatal day 200. It means, with the increasing age, smoke exposed offspring gained weight more rapidly as compared to those in the sham group.

3.1.3 Glomerular fraction area

3.1.3.1 Glomerular fraction area on postnatal day 16

Numeric comparison between the two groups is provided in Table 3.5.

Table 3.5 Numeric comparison of glomerular fraction area (micro-meter square) between the smoke and sham groups, on postnatal day 16

Group	Number of offspring	Mean (μm^2)	SD
Smoke	8	0.042	0.014
Sham	8	0.061	0.020

As shown in Table 3.5, on postnatal day 16, smoke exposed offspring had lower mean glomerular fraction area and standard deviation as compared to offspring in the sham group.

Manova was performed and result was significant ($F_{1, 14} = 4.78$, $p = 0.05$). It means, on postnatal day 16, glomerular fraction area of offspring in both the groups was significantly different.

3.1.3. 2 Glomerular fraction area on postnatal day 200

Table 3.6 provides a summary of numeric comparison between the two groups.

Table 3.6 Numeric comparison of glomerular fraction area (micrometer square) between the smoke and sham groups, on postnatal day 200

Group	Number of offspring	Mean (μm^2)	SD
Smoke	8	0.031	0.006
Sham	8	0.036	0.012

As shown in Table 3.6, there was not much difference in mean glomerular fraction area between the two groups.

Manova was performed. The test result was insignificant ($F_{1, 14}$ = 0.1.18, p = 0.3). It means glomerular fraction area of offspring was not significantly different between the groups, on postnatal day 200.

3.1.4 Cortical fraction area

3.1.4.1 Cortical fraction area on postnatal day 16

Table 3.7 provides a summary of numeric comparison on this variable between the two groups.

Table 3.7 Numeric comparison of cortical fraction area (micro-meter square) between the smoke and sham and groups, on postnatal day 16

Group	Number of offspring	Mean (μm^2)	SD
Smoke	8	0.668	0.09
Sham	8	0.653	0.15

As shown in Table 3.7, mean cortical fraction area in both the groups was similar on postnatal day 16.

Manova was performed and test result was insignificant ($F_{1, 14}$ = = 0.05, p = 0.82). Therefore, mean cortical fraction area of offspring, in both the groups, was not significantly different on postnatal day 16.

3.1.4.2 Cortical fraction area on postnatal day 200

Table 3.8 provides a summary of numeric comparison between the smoke and sham groups.

Table 3.8 Numeric comparison of cortical fraction area (micrometer square) between the smoke and sham groups, on postnatal day 200

Group	Number of offspring	Mean (μm^2)	SD
Smoke	8	0.802	0.12
Sham	8	0.619	0.13

As shown in Table 3.8, mean cortical fraction area was more in the smoke group as compared to the sham group.

On comparison between Table 3.7 and 3.8, it could be observed that cortical fraction area increased with growth, in the smoke group while there was almost no change in the sham group.

The result of Manova was significant ($F_{1, 14} = 8.1$, $p = 0.01$). Therefore, mean cortical fraction area was significantly different between smoke and sham groups on postnatal day 200. It means with the growth, prenatal exposure to smoke caused significant change in cortical fraction area.

3.1.5 Ratio between glomerular and cortical fraction areas

3.1.5.1 Ratio between glomerular and cortical fraction areas on postnatal day 16

Descriptive statistics for both the groups is given in Table 3.9. Histological details of glomeruli in the cortex of both the groups, on postnatal day 16, are provided in Figures 3.1 and 3.2.

Table 3.9 Numeric comparison of ratio between glomerular and cortical fraction areas between the smoke and sham groups, on postnatal day 16

Group	Number of offspring	Mean	SD
Smoke	8	0.063	0.02
Sham	8	0.093	0.02

As shown in Table 3.9, mean ratio between glomerular and cortical fraction area was 1.5 times more in the sham group as compared to the smoke group. However, data was evenly spread in both the groups.

Manova was performed and test result was significant ($F_{1, 14} = 9.31$, $p = 0.01$). Therefore, mean ratio between glomerular and cortical fraction areas, in both the groups, was significantly different on postnatal day 16. It means prenatal

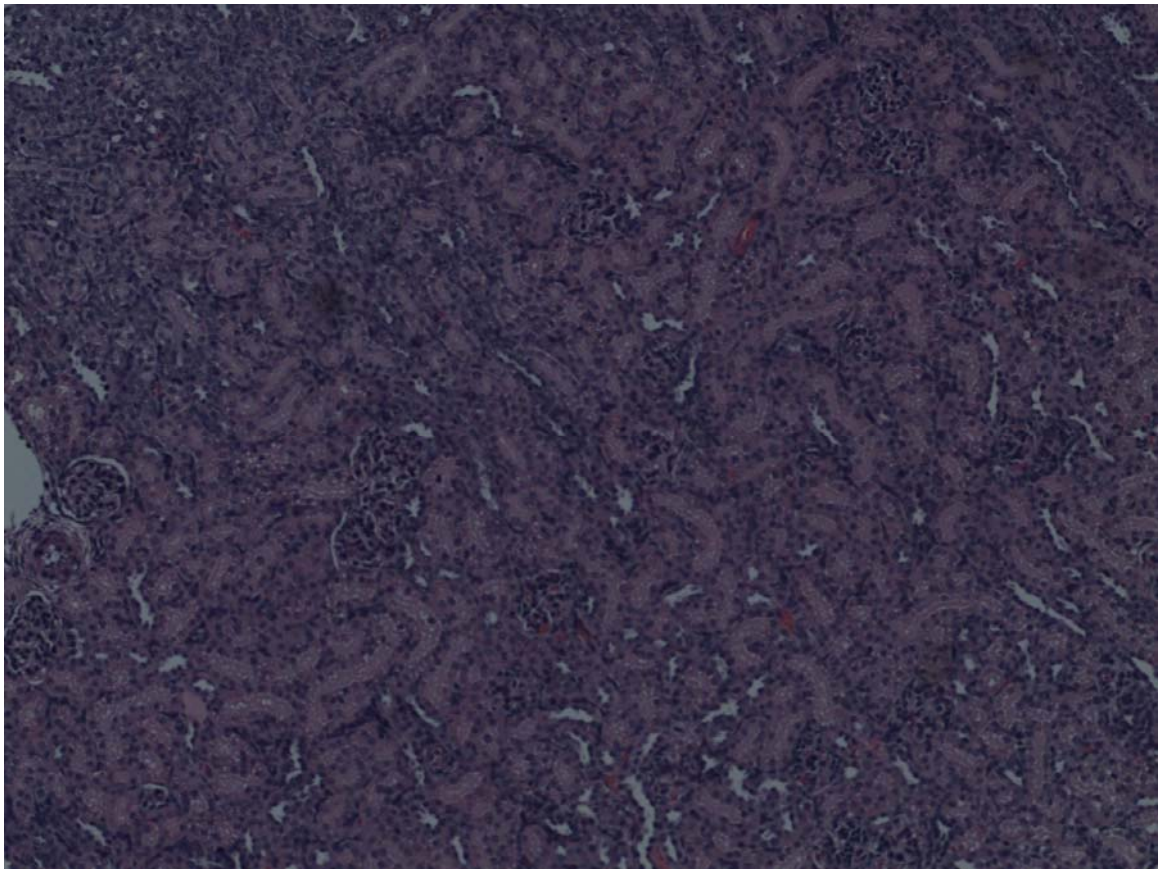

Figure 3.1 Glomeruli in the cortex of prenatal smoke group rats, on postnatal day 16

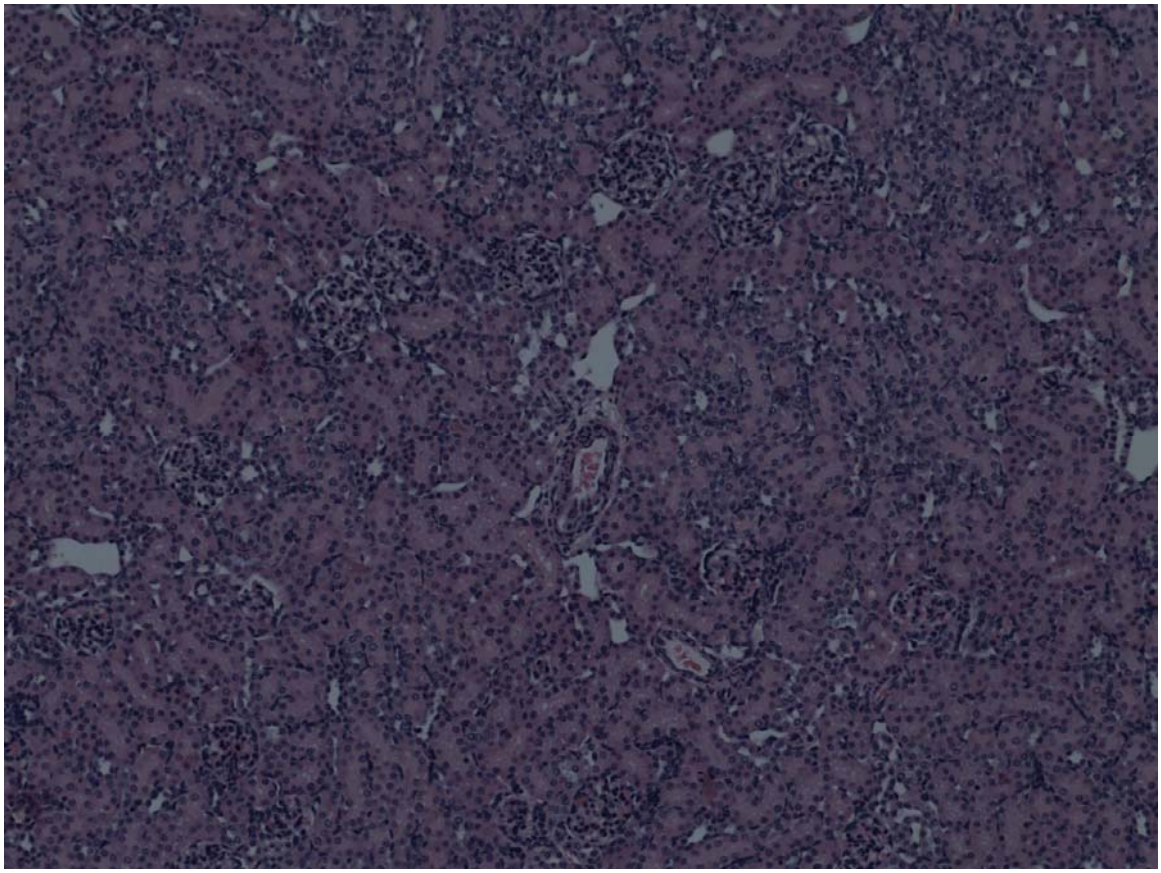

Figure 3.2 Glomeruli in the cortex of sham group rats, on postnatal day 16

exposure to smoke significantly changed the mean ratio between glomerular and cortical fraction areas on postnatal day 16.

3.1.5.2 Ratio between glomerular and cortical fraction areas on postnatal day 200

Descriptive statistics for both the groups is given in Table 3.10. Histological details of glomeruli in the cortex of both the groups, on postnatal day 200, have been provided in Figures 3.3 and 3.4.

Table 3.10 Numeric comparison of ratio between glomerular and cortical fraction areas between the smoke and sham groups, on postnatal day 200

Groups	Number of offspring	Mean	SD
Smoke	8	0.04	0.008
Sham	8	0.06	0.011

As shown in Table 3.10, mean ratio was 1.5 times higher in the sham group as compared to the smoke group. Standard deviation was also lower in the smoke group as compared to the sham group.

On comparison between Table 3.9 and 3.10, it could be concluded that ratio between glomerular and cortical fraction areas per kidney decreased with

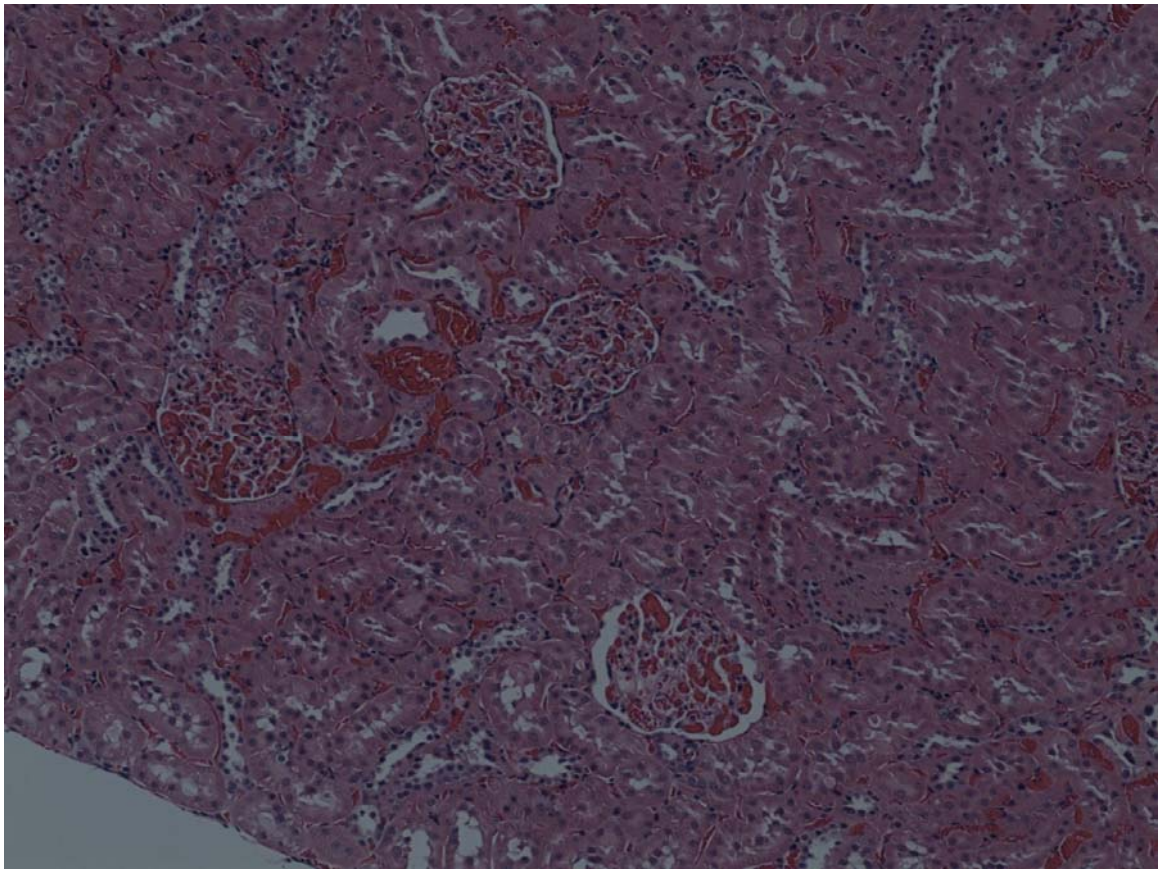

Figure 3.3 Glomeruli in the cortex of prenatal smoke exposed rats, on postnatal day 200

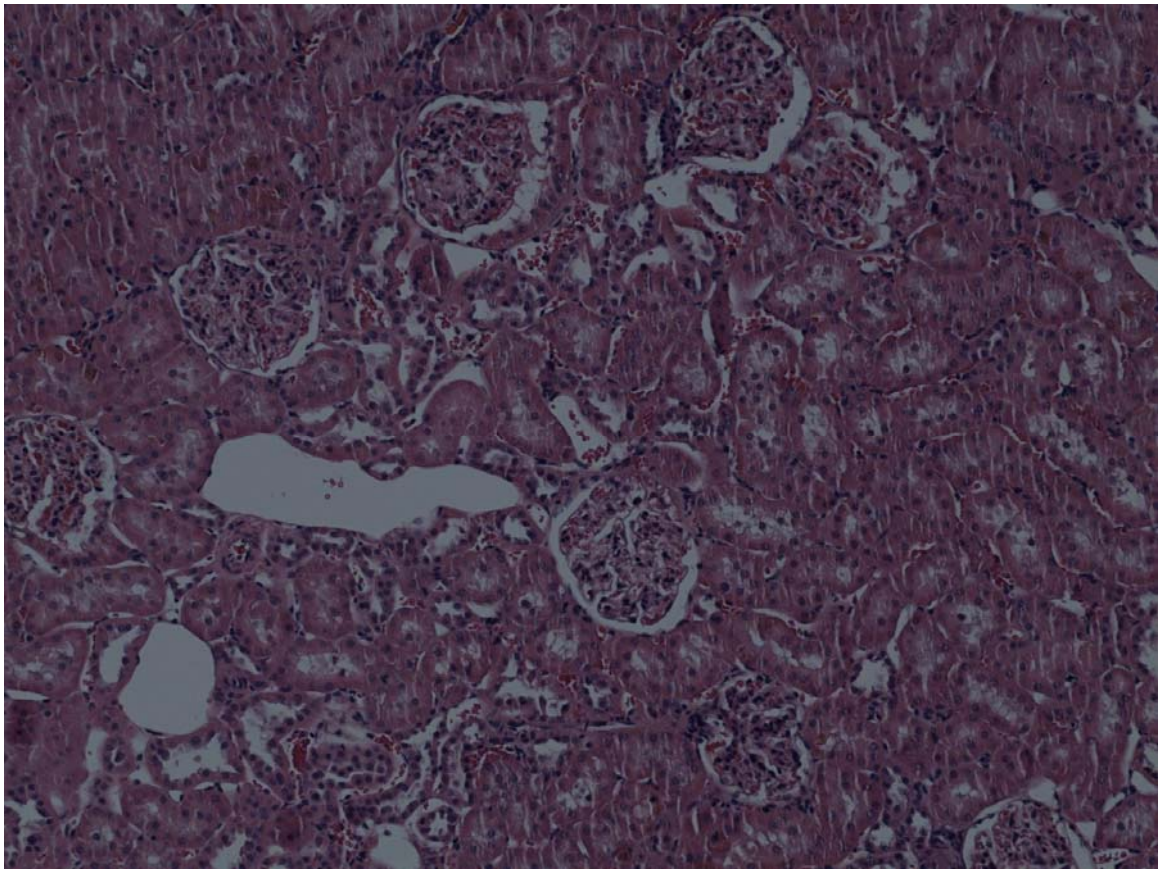

Figure 3.4 Glomeruli in the cortex of sham group rats, on postnatal day 200

the growth in both the groups. However, ratio remained 1.5 times higher in the sham group as compared to the smoke group on postnatal day 200.

Manova was performed. The test result was significant ($F_{1, 14}$ = 14.82, p =0.002). Therefore, mean ratio between glomerular and cortical fraction areas in both the groups was significantly different on postnatal day 200. It means prenatal exposure to smoke significantly changed the mean ratio between glomerular and cortical fraction areas on postnatal days 200. Therefore, the significant effect of prenatal smoke exposure on this ratio persisted with age, from postnatal day 16 to 200.

3.2 Results related to secondary objective

Secondary aim of the current study was to investigate changes in blood pressure during adulthood in male offspring of rats exposed to cigarette smoke during pregnancy. The results are provided in the following lines.

3.2.1 Diastolic Blood Pressure

Numeric description for diastolic blood pressure, on postnatal days 160 and 200, is provided in Table 3.11.

Table 3.11 Numeric comparison of diastolic blood pressure (mm Hg) between the smoke and sham groups

Postnatal day	Group	Number of offspring	Mean	SD
160	Smoke	7	92.07	4.08
	Sham	6	85.42	7.56
200	Smoke	7	89.80	4.62
	Sham	6	79.64	10.7

As shown in Table 3.11, mean diastolic blood pressure in the smoke group was more than 90 mm Hg on postnatal day 160 and almost equal to 90 mm Hg on postnatal day 200. It was higher in the smoke group as compared to the sham group. A decrease in mean diastolic blood pressure was also noted from day 160 to 200 in both the groups. Standard deviation was higher in the sham group as compared to the smoke group.

Manova was performed. The test result was insignificant ($F_{1, 11} = 4.07$, $p = 0.07$) on postnatal day 160 and significant ($F_{1, 11} = 5.24$, $p = 0.04$) on postnatal day 200. Therefore, mean diastolic blood pressure was not significantly different on postnatal days 160 but significantly different on postnatal day 200 between the smoke and sham groups. It means exposure to smoke significantly increased the mean diastolic blood pressure on postnatal day 200 (although within normal limits).

3.2.2 Systolic Blood Pressure

Numeric description for both the groups is provided in Table 3.12.

Table 3.12 Numeric comparison of systolic blood pressure (mm Hg) between the smoke and sham groups, on postnatal days 160 and 200

Postnatal day	Group	Number of offspring	Mean	SD
160	Smoke	7	130.41	5.46
	Sham	6	125.95	7.06
200	Smoke	7	130.19	6.63
	Sham	6	119.96	8.11

As shown in Table 3.12, mean systolic blood pressure of smoke exposed offspring was more than 130 mm Hg on postnatal days 160 and 200. It was also higher in the smoke group as compared to the sham group on postnatal days 160 and 200. A decrease in mean systolic blood pressure was also noted from postnatal day 160 to 200 in both the groups. However, this decrease was more visible in the sham group as compared to the smoke group.

Manova was performed. The test result was insignificant ($F_{1, 11} = 1.45$, $p = 0.23$) on postnatal day 160 and significant ($F_{1, 11} = 6.28$, $p = 0.03$) on postnatal day 200. Therefore, mean systolic blood pressure was not significantly different on postnatal days 160 but significantly different on postnatal day 200 between

the smoke and sham groups. It means exposure to smoke significantly increased the mean systolic blood pressure on postnatal day 200.

3.2.3 Pulse Pressure

Numeric description for both the groups is provided in Table 3.13.

Table 3.13 Numeric comparison of pulse pressure (mm Hg) between the smoke and sham groups, on postnatal days 160 and 200

Postnatal day	Group	Number of offspring	Mean	SD
160	Smoke	7	38.35	3.30
	Sham	6	40.51	3.40
200	Smoke	7	40.10	4.03
	Sham	6	40.21	3.23

As shown in Table 3.123, mean pulse pressure was similar on postnatal days 160 and 200 between the smoke and sham groups.

Manova result was also insignificant on postnatal days 160 ($F_{1, 11} = 1.34$, $p = 0.27$) and 200 ($F_{1, 11} = 0$, $p = 1$). Therefore, mean pulse pressure was not significantly different between the two groups, on postnatal days 160 and 200.

3.2.4 Mean blood pressure

Numeric description is provided in Table 3.14.

Table 3.14 Numeric comparison of mean blood pressure (mm Hg) between the smoke and sham groups, on postnatal days 160 and 200

Postnatal day	Group	Number of offspring	Mean	SD
160	Smoke	7	110	4.9
	Sham	6	104	7.3
200	Smoke	7	108	5.6
	Sham	6	98	9.4

As shown in Table 3.14, mean blood pressure was higher and data was less widely spread in the smoke group as compared to the sham group on postnatal days 160 and 200.

Manova result was insignificant on postnatal days 160 ($F_{1, 11} = 2.78$, $p = 0.12$) but significant on postnatal day 200 ($F_{1, 11} = 5.88$, $p = 0.03$). Therefore, mean blood pressure was significantly different between the two groups, on postnatal days 160 and 200.

3.2.5 Heart rate

Numeric description for both the groups is provided in Table 3.14.

Table 3.15 Numeric comparison of heart rate (beats/minute) between the smoke and sham groups, on postnatal days 160 and 200

Postnatal day	Group	Number of offspring	Mean	SD
160	Smoke	7	311.86	14.76
	Sham	6	305.84	14.74
200	Smoke	7	309.98	12.45
	Sham	6	289.49	14.12

As shown in Table 3.14, mean heart rate was almost similar in both the groups on postnatal days 160 but there was difference of 20 heart beats/minute between the smoke and sham groups on postnatal day 200.

Manova result was insignificant ($F_{1, 11} = 0.54$, $p = 0.48$) on postnatal days 160 but significant ($F_{1, 11} = 7.74$, $p = 0.018$) on postnatal day 200. Therefore, mean heart rate was significantly different between the two groups, on postnatal day 200.

3.2.6 Diurnal variations in blood pressure

3.2.6.1 Diurnal diastolic blood pressure

Graphic presentation on this variable is provided in Figures 3.5 and 3.6. As shown in figure 3.5, mean diastolic blood pressure was higher in the smoke group in the morning while it was higher in the evening in the sham group on postnatal day 160. Statistical analysis showed insignificant ($p = 0.08$) change in the smoke group but significant ($p = 0.02$) in the sham group. Therefore, there was significant diurnal variation in mean diastolic blood pressure in the sham group.

As shown in Figure 3.6, similar observations could be made regarding mean diastolic blood pressure, on postnatal day 200. Manova results showed significant diurnal variation in mean diastolic blood pressure in the smoke ($p = 0.04$) and in the sham ($p = 0.01$) groups.

3.2.6.2 Diurnal systolic blood pressure

Figures 3.7 and 3.8 provide information on diurnal variation in systolic blood pressure in both the groups.

As shown in Figure 3.7, mean systolic blood pressure was slightly higher in the morning as compared to evening in the smoke group while it was almost no diurnal variation in the sham group, on postnatal day 160. Manova results

were also insignificant in the smoke (p = 0.19) and sham (p = 0.89) groups, on postnatal days 160.

As shown in Figure 3.8, similar observations were made for systolic blood pressure on postnatal day 200. Test results were insignificant for group but significant for the sham (p = 0.02) group.

3.2.6.3 Diurnal mean blood pressure

Graphic description on this variable, for both the groups, is provided in Figures 3.9 and 3.10.

As shown in Figures 3.9 and 3,10, mean blood pressure was higher in the smoke group in the morning hours while in the evening it was higher in the sham group on postnatal days 160 and 200. Manova was performed. The test result was insignificant on postnatal days 160 in the smoke (p = 0.19) and sham groups (p = 0.27). However, on postnatal day 200, the test result was insignificant in the smoke (p = 0.06) group but significant (p = 0.008) in the sham group.

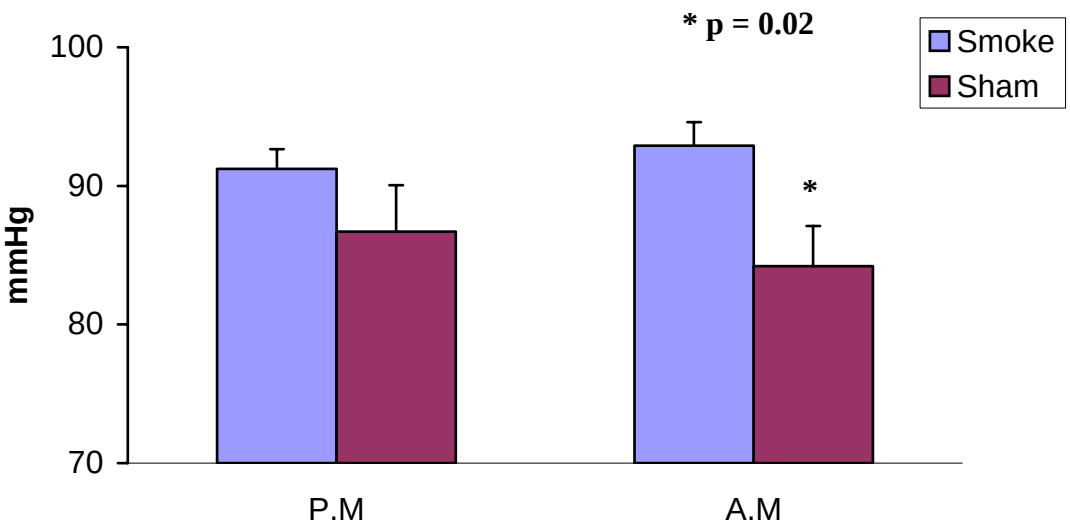

Figure 3.5 Comparison of diurnal variations in diastolic blood pressure (mm Hg) in the smoke and sham groups, on postnatal days 160

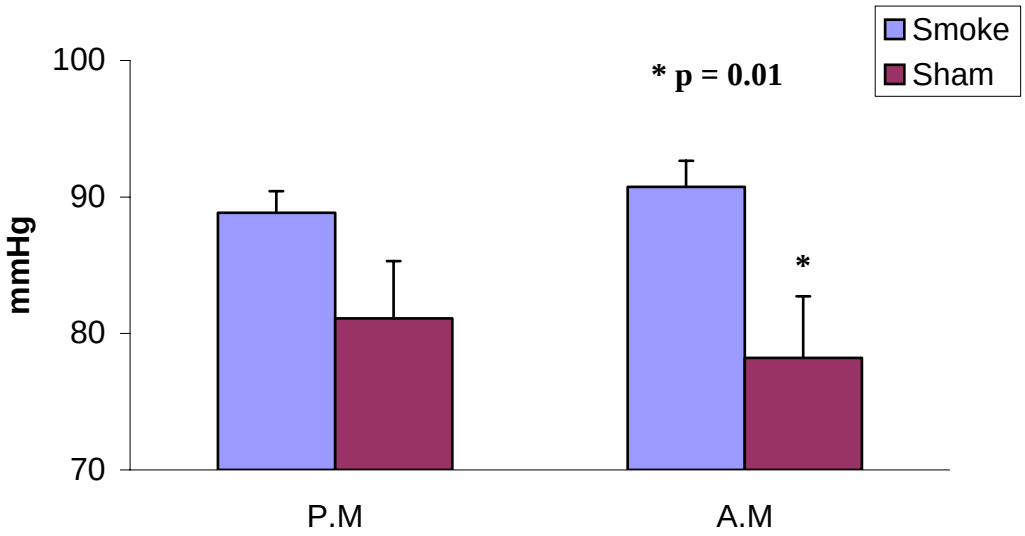

Figure 3.6 Graphic comparison of diurnal variation in diastolic blood pressure (mm Hg) in the smoke and sham groups, on postnatal day 200

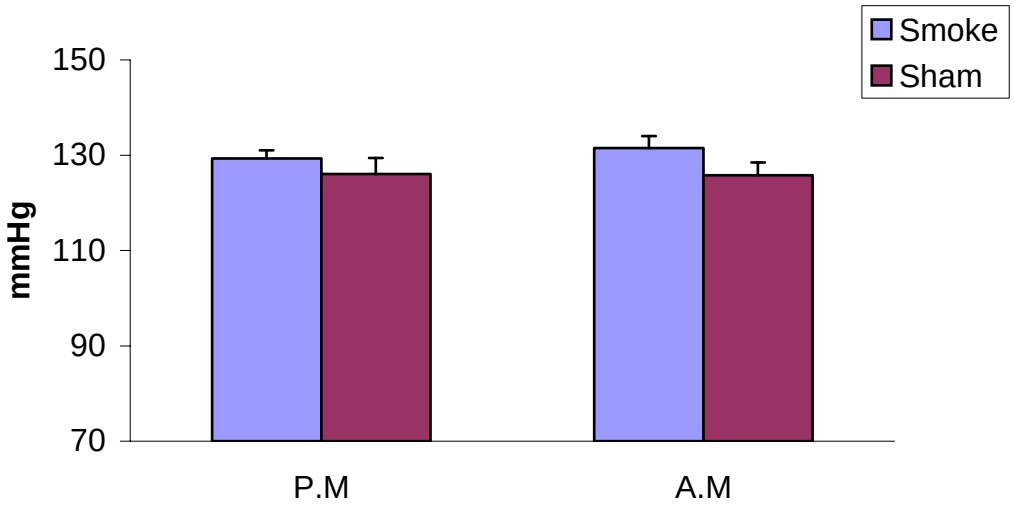

Figure 3.7 Comparison of diurnal variation in systolic blood pressure (mm Hg) in the smoke and sham groups, on postnatal days 160

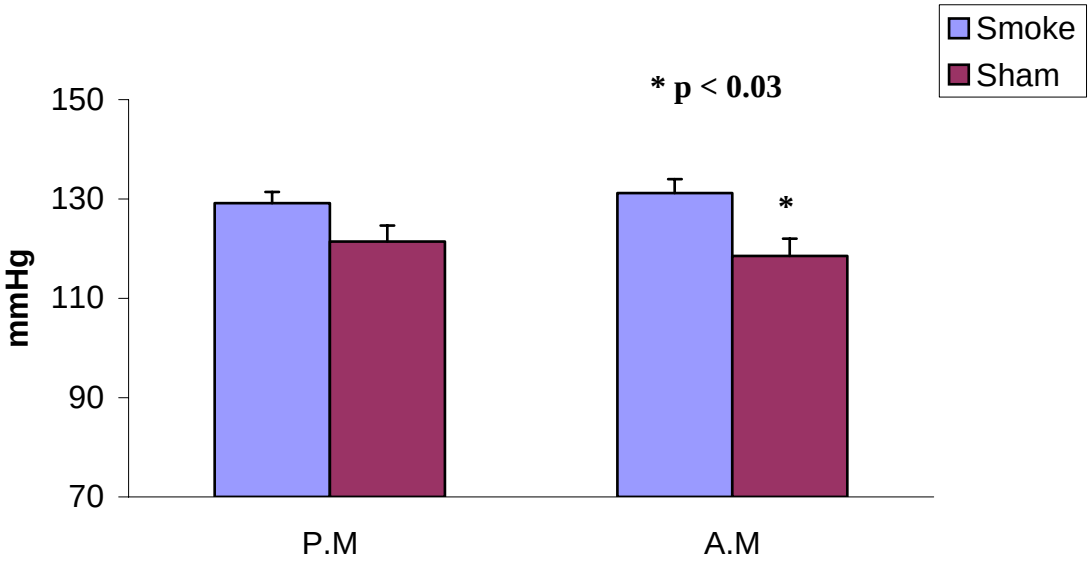

Figure 3.8 Comparison of diurnal variation in systolic blood pressure (mm Hg) in the smoke and sham groups, on postnatal days 200

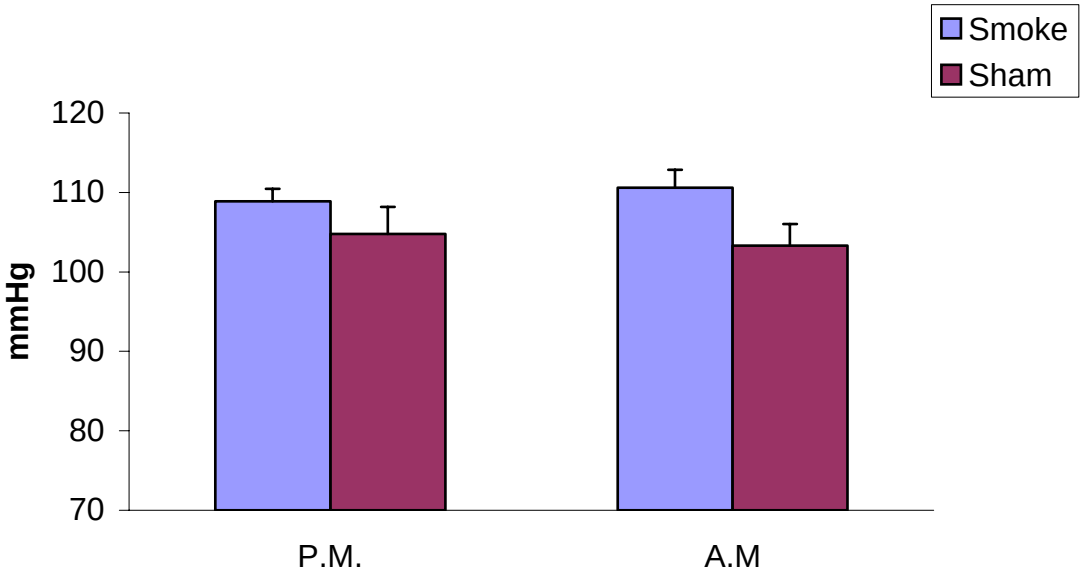

Figure 3.9 Comparison of diurnal variation in mean blood pressure (mm Hg) between the smoke and sham groups, on postnatal days 160

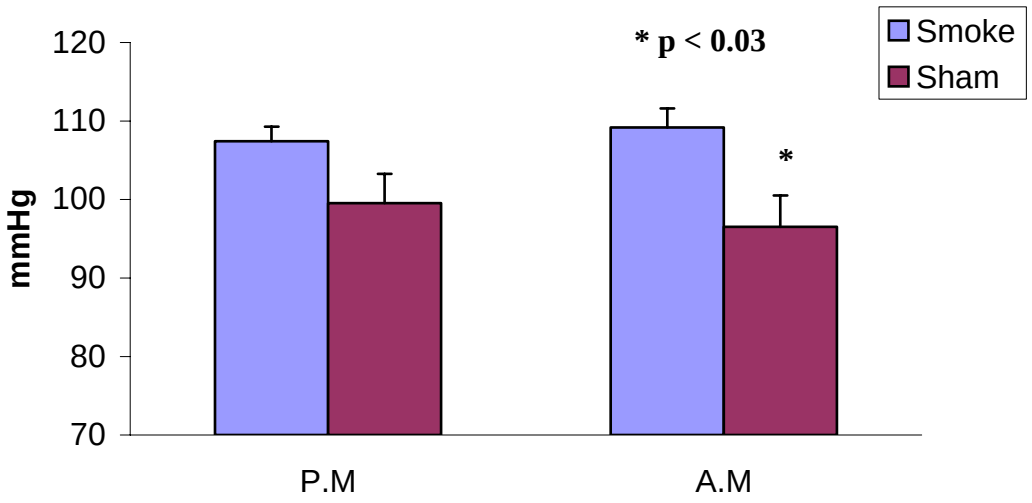

Figure 3.10 Comparison of diurnal variation in mean blood pressure (mm Hg) between the smoke and sham groups, on postnatal days 200

3.2.7 Percent change in diurnal variation in blood pressure

Percent change in diurnal variation of blood pressure is graphically presented in Figures 3.11 and 3.12. The observed differences were evaluated through two-sample *t* test for independent variables. The results are provided in Table 3.16.

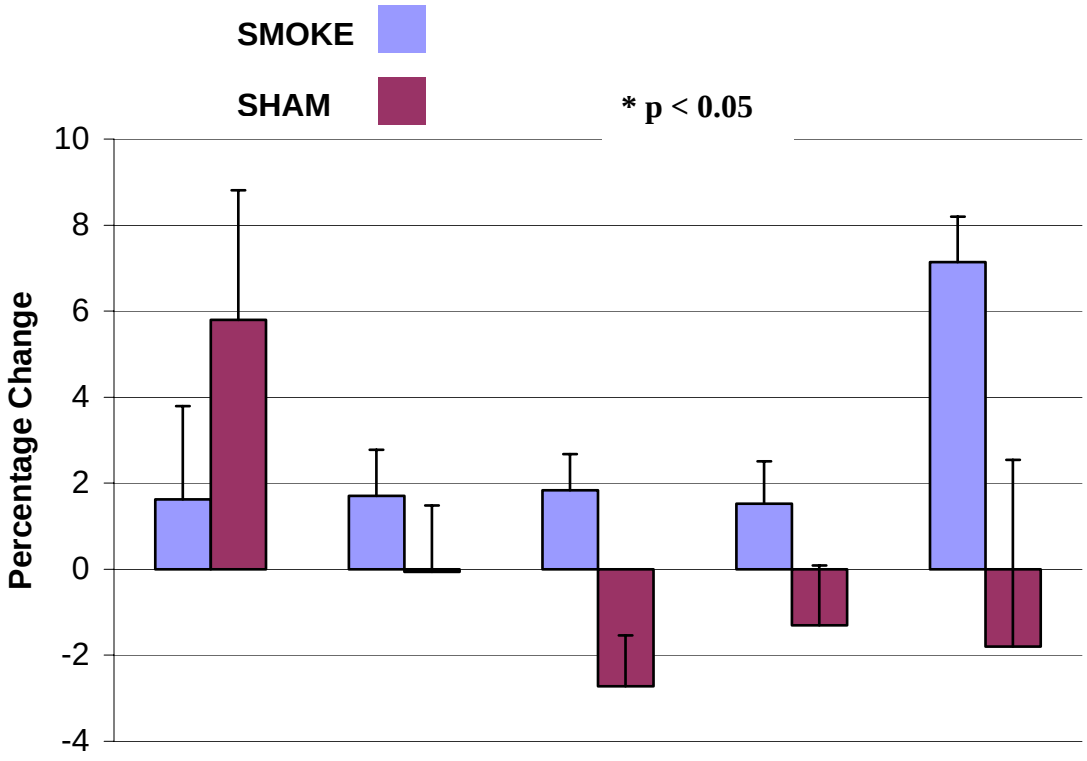

Figure 3.11 Percent change in diurnal variation of blood pressure (mm Hg) between the smoke and sham groups, on postnatal day 160

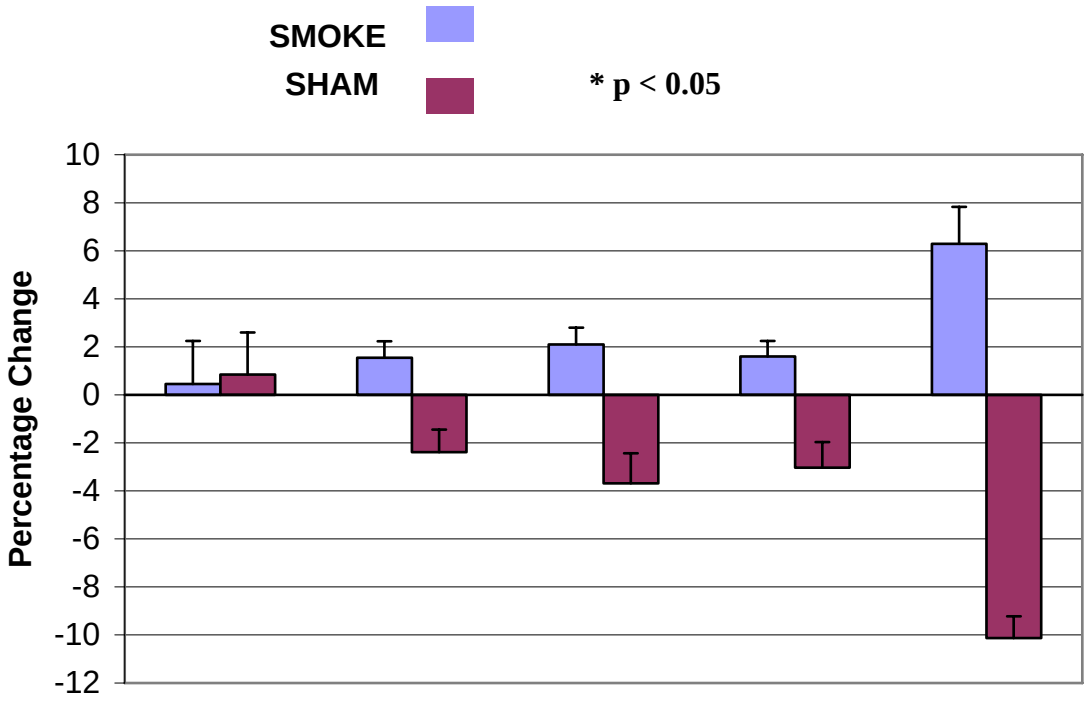

Figure 3.12 Percent change in diurnal variation of blood pressure (mm Hg) between the smoke and sham groups, on postnatal day 200

Table 3.16 Percent change in diurnal variation in blood pressure in the smoke and sham groups, on postnatal days 160 and 200

Postnatal day	Variables	Mean difference	p	95% Confidence Interval
160	Systolic blood pressure	4.56	0.01	1.42 – 7.68
	Diastolic blood pressure	1.77	0.35	-2.26 – 5.81
	Mean blood pressure	2.82	0.12	-0.83 – 6.49
	Pulse pressure	-4.17	0.28	-12.17 – 3.83
	Heart rate	8.94	0.05	-0.19 - 18
200	Systolic blood pressure	3.93	0.005	1.42 – 6.43
	Diastolic blood pressure	5.78	0.002	2.74 – 8.82
	Mean blood pressure	4.63	0.003	1.99 – 7.27
	Pulse pressure	-0.39	0.88	-5.9 – 5.18
	Heart rate	16.42	<0.001	12.29 20.55

As shown in Table 3.16, percent change in diurnal variation in systolic blood pressure was significant on postnatal days 160 and 200 while for diastolic and mean blood pressures, percent change in diurnal variation was only significant on postnatal day 200. Similarly, percentage change in mean blood pressure and heart rate was also significant on postnatal day 200

Chapter Four: Discussion

4.1 General discussion

The current study aimed to investigate the morphological and histological changes in kidney and effect on blood pressure among male rats, at adulthood, secondary to prenatal cigarette smoke exposure.

The results presented here show that prenatal exposure to cigarette smoke has significant effects on kidney morphology and blood pressure. It concludes that maternal smoking during pregnancy significantly decreases the mean ratio between glomerular and cortical fraction areas, on postnatal day 16. This ratio remained significantly decreased in the smoke group, on postnatal day 200. The results thus provide evidence that prenatal smoke exposure caused permanent decrease in mean ratio between glomerular and cortical fraction areas. The present study further concludes that prenatal exposure to smoke significantly reduces kidney weight of rats on postnatal day 16. However, mean kidney weight in both the groups was not significantly different on postnatal day 200, thereby explaining that with the increasing age, smoke exposed offspring gained weight more rapidly as compared to those in the sham group. Body weight of each offspring in both groups was also recorded as an indicator of change in postnatal growth and to compare any change in body weight with variation in kidney weight. The results show that prenatal exposure to smoke significantly reduced mean body weight of rats on postnatal day 16. There was,

however no significant difference in body weight on postnatal day 200. It provides evidence that with the increasing age, smoke exposed offspring gained body weight more rapidly than the sham group. As for as findings related to change in blood pressure are concerned, the study results show a significant increase in mean systolic and diastolic blood pressures and heart rate on postnatal day 200 in rats exposed to prenatal cigarette smoke. However, this increase in blood pressure and heart rate was insignificant on postnatal day 160. It shows that alterations in the kidney morphology could be among the factors responsible for the changes in blood pressure.

The literature was reviewed to compare the results of the current study with previous findings in the literature. On comparison, there were many similarities, a few differences and new knowledge was contributed to the perinatal literature.

Many epidemiological and experimental studies have suggested that intrauterine environment plays an extremely important role in determining the future health of an individual. Genetic and lifestyle factors further enhance this 'programming' effect of an adverse intrauterine environment (1,2). Kidney is considered one of the major organs, which is affected by an unfavourable prenatal environment (73). The findings of the recent study have also shown that prenatal exposure to cigarette smoke caused deleterious effect on kidney growth.

In an epidemiological study, Lampl et al. observed the impact of prenatal smoke exposure on morphology of kidney and heart. They found significant difference in kidney width, thickness, length and volume during second and third trimester between smoking and nonsmoking mothers. Length and width of the kidney was proportionately thinner in fetus exposed to prenatal cigarette smoke. However, changes in the cardiac growth were reverse and compensatory enlargement in cardiac size occurred after 30 weeks of gestation. It was also suggested that an increase in the size could be due to maladaptive changes caused by hypoxia and other chemicals present in cigarette smoke (107). The findings of the current study have shown similar changes in the growth of kidney. Prenatal exposure to smoke significantly reduced kidney weight of rats after birth. With increasing age, smoke exposed rats gained weight more rapidly as compared to unexposed rats perhaps through a compensatory mechanism.

The association between maternal smoking and fetal growth retardation was first time described in 1957, and these results have since been confirmed by many other studies. For example, Ventura et al., in 2003, showed that incidence of low birth weight was twice among singleton infants exposed to prenatal cigarette smoke as compared to those born to non-smokers (49). In another study, the same association was analyzed in both sexes and the cigarette smoke exposure was observed to reduce the birth weight in male fetus more than in the female fetus (51). Polanska et al. in their study published in 2004, showed that risk for intrauterine growth retardation increased with the number of cigarettes

smoked indicating a dose dependent effect (108). In a review article by Zhang et al., in 2005, it was concluded that there was enough evidence that fetal exposure to hypoxia, alcohol, tobacco smoking, and cocaine may cause in-utero programming leading to an increased risk of adulthood diseases. Chronic hypoxia during pregnancy was thought to result in fetal intrauterine growth retardation (109). The findings of the current study also correspond to the literature, in that prenatal exposure to smoke significantly reduced mean body weight of male rats at birth. However, our study provides no evidence on dose response affect. In the present study, we also found no significant difference in body weight on postnatal day 200 between the smoke and sham groups. It means smoke exposed offspring improved their body weight more rapidly than the sham group. A recent study by Wideroe et al. provides an explanation for this result. It was shown that smoking during pregnancy was a risk factor for the development of childhood overweight. However, the risk of overweight associated with smoking during pregnancy was independent of intrauterine growth retardation (110).

Many researchers have investigated the role of maternal nutritional deficiency in the development of adulthood diseases (78,79,80,81). Ingelfinger et al., in their review article published in 2004, discussed the factors that initiated perinatal programming and caused renal and cardiovascular diseases. It was concluded that compromised maternal nutrition in intrauterine life causes changes in the renal tubular transport system, which may then be responsible for

altered renal function (111). Maternal hyperglycemia, vitamin A and exposure to drugs have been associated with variations in nephron number in other studies (78). However, Armitage et al. concluded on stereology, in 2005, that lard-rich diet during pregnancy caused no differences in kidney weight, glomerular number or volume as compared to rats on control diet. Adult rats on lard-rich diet, however, showed increase in aortic stiffness and reduced endothelium-dependent relaxation (112).

The current literature provides limited information on morphological and histological changes in kidney at adulthood as a consequence of exposure to maternal smoking during pregnancy. However, the results of our study provide comprehensive information on this association. The data presented in study demonstrates that mean ratio between glomerular and cortical fraction areas decreases significantly after birth due to prenatal exposure to smoke. We further observed that this change in the ratio between glomerular and cortical fraction areas persisted in the adulthood, thereby providing evidence on fetal programming for nephrogenesis secondary to prenatal cigarette smoke exposure.

An association between low birth weight and adult-onset diseases could provide basis for the above result. Mitchell et al., in 2004, conducted a study on fetal sheep that had retarded intrauterine growth due to natural twining. They used stereological techniques to estimate glomerular capillary length and

filtration surface area. The results showed that nephron endowment was 40% lower in twin fetuses as compared to controls. The researchers concluded that intrauterine growth retardation, due to twinning caused reduced nephron endowment, while late gestational intrauterine growth retardation did not have such effect. It was, therefore, suggested that reduction in nephron endowment was dependent on the timing of the growth restriction (113).

In a number of previous studies, low protein diet was shown to produce deleterious effects on kidneys. Vehaskari et al. observed that the kidney and body sizes of the offspring decreased at birth when pregnant Sprague-Dawley rats were kept on low-protein diet. Total number of glomeruli per kidney decreased by 28% in males and 29% in females. By the age of eight weeks, both male and female low-protein pregnant rats had systolic blood pressures that were 20 to 25 mm Hg higher than those of control animals (77). Woods et al. in their experiment showed that low-protein diet to pregnant rats throughout gestation reduced birth weight and kidney/body weight ratio. It was proposed that perinatal protein restriction in the rats suppressed the new born intrarenal renin-angiotensin system and led to reduced number of glomeruli, glomerular enlargement, and hypertension in the adult (81). Results of the present study showed almost same effect but risk factor was prenatal exposure to cigarette smoke instead of low protein diet.

The results of the current study also show that prenatal exposure to smoke caused significant increase in mean systolic and diastolic blood pressure on postnatal day 200. A significant increase in mean heart rate was also noticed on same postnatal day secondary to prenatal exposure to smoke. Previous studies provide evidence that low birth weight and low nephron numbers could be among the risk factors for the cardiovascular disease in later life (107) as it was observed in the current study. Likewise, studies have shown an association between adverse intrauterine environment and hypertension in the adulthood (3, 80, 109).

Different researchers have explained the relationship between adverse intrauterine environment and development of hypertension in the adulthood. Different mechanisms have also been proposed to explain this association. Steuerer et al. proposed oxidative stress to be a contributing factor (68), whereas In another study, intrauterine undernutrition was considered to be the cause of endothelial dysfunctional development (69). Lackland et al. concluded reduced nephrogenesis, greater susceptibility to progressive renal disease, impaired development of endothelium, and increased sensitivity to glucocorticoids for this relationship (65). Woods et al. observed that the renin-angiotensin system was central in setting the trajectory that led to cardiovascular disease particularly hypertension (74). In one study, primary sodium retention and expanded extracellular volume were found critical factors during the development of hypertension (76). Woods et al. in another study proposed that prenatal protein

restriction suppressed intrarenal renin-angiotensin system and caused less number of glomeruli, glomerular enlargement, and hypertension in the adult rats (81). Fogo et al. suggested that interactions of the renin-angiotensin system with aldosterone and bradykinin might have had impact on both blood pressure and tissue injury (82). In a study by Langley-Evans et al. it was clear that fetal exposure to glucocorticoids of maternal origin was the first step in the programming of hypertension and coronary heart disease (95). In a study published in 2005, deficiency in endothelial nitric oxide synthetase has been proposed as an underlying factor in the development of adulthood vascular dysfunction secondary to intrauterine insult (115).

4.2 Strengths

As already explained, role of many risk factors in fetal programming of hypertension and renal diseases have been discussed by various researcher but limited information is available on histological changes in kidney taking place at adulthood secondary to prenatal maternal smoking. The current study, however, provides comprehensive information on this topic demonstrating that prenatal exposure to smoke causes significant decrease in mean ratio between glomerular and cortical fraction areas after birth. It was also shown that this change in the kidney persists in adulthood, thereby providing evidence on fetal programming for nephrogenesis secondary to prenatal cigarette smoking.

In the current study, both the comparative groups were kept similar, except smoke exposure. Rats of the same sex were selected for both groups to control the confounding effect of sex. Providing air puffs of similar volume and number to the sham group as in the smoke exposed equalized stress caused by the smoke exposure system in the smoke group. Similarly, rats in both groups had free access to food and water. The rats in the smoke group were exposed to a pre-determined amount of cigarette smoke that could be helpful in the assessment of quantity of smoke exposure causing deleterious effect on body organs like kidney and cardiovascular system.

Wistar Kyoto rats were selected for this study mainly because of the fact that this strain has identical blood pressure to humans. This similarity in the level of blood pressure made it convenient to compare the findings of this study comparable with that of humans.

Data was collected through unbiased computer based soft ware programs like telemetry system, Stereo Investigator Program and morphometry. Use of operational definitions was another important step towards accurate measurement of all the variables in this study.

2.3 Limitations

As already stated in section on Methodology, one offspring from each liter in both the groups was randomly selected and sacrificed for removal of kidneys

on postnatal day 16. Similarly, on postnatal day 140, one offspring from each liter in both the groups was randomly selected and telemetry system was implanted in the abdominal cavity and a probe was placed in the femoral artery to measure blood pressure on postnatal days 160 and 200. On postnatal day 200, implant and probe were removed and rats were sacrificed to remove kidneys. Therefore, although rats were selected from the same litter but measurement for kidney size and histological changes were made on different rats. Therefore, role of any individual variation on the study results could not be ruled out. However, changes in blood pressure and kidney histology were observed on same rats on postnatal day 200.

Data was collected on rats for a long period during this study. Even after surgical procedures rats were followed up to postnatal day 200. During this period, some rats became sick while others died. Data of the sickness period was missing. For example, information on blood pressure could be recorded on thirteen rats because three rats died during the course of the study.

Rat is a nocturnal animal therefore we analyzed the diurnal variations in blood pressure. As limited knowledge is available on diurnal variations in blood pressure secondary to cigarette smoke exposure, any impact of such variations on the final results related to changes in blood pressure could not be ruled out in the current study.

As stated previously, data was collected for a long time and certain measurements like blood pressure and heart rate were recorded every five minutes. Statistical packages were used at each stage of data collection and although rigorous techniques were use, the possibility of any random error and its impact on the study results could not be ruled out.

2.4 Recommendations

The main finding of the current study comprehensively explains the role of maternal smoking during pregnancy in the development of altered renal functions in neonatal period and at adulthood. We believe that this finding has opened a new chapter for the researchers working in the area of fetal programming. As there is limited information in the present literature on the effect of prenatal exposure on ratio between glomerular and cortical fraction areas, additional research is required to confirm the key finding of the current study.

The lessons we learned from this study can be used for future research especially at designing and execution stages of study. We recommend that future studies should be conducted on a larger sample size in order to see whether findings of the current study remain the same. Although smoke exposure system and telemetry systems are designed for eight animals in each group, it can be managed by arranging more than one set of animals and exposing them to the system with short intervals.

Another point we would like to recommend is that serial section method of the Stereo- Investigator Program should be used in addition to fraction-fractionators for morphometric measurements to see the histological changes in more detail. If it is not possible to get all the facilities in one lab, work may be extended with the collaboration of other laboratories. Similarly, in the current study H&E staining was used for morphometry, thus precluding the analysis of changes at cellular level in this program. Therefore, we believe that future investigators should also arrange other staining procedures and try to observe glomerular changes in more detail through electron microscopy.

Another issue related to the present study is the current smoking practices and activities of public health department for the prevention of maternal smoking during pregnancy. As already stated, percentage of mothers smoking during pregnancy is high in Canada in spite of the fact that smoking is a preventable risk factor. We believe that more organized efforts at community level, government level and political level are required to motivate mothers to quit smoking during pregnancy. We also believe that research in this field may be promoted to explore genetic factors related to smoking.

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