PRENATAL IRRADIATION EFFECTS ON MOUSE CARDIOVASCULAR SYSTEM

PRENATAL IONIZING RADIATION EXPOSURE EFFECTS ON CARDIOVASCULAR HEALTH AND DISEASE IN C57Bl MICE

By

SHAYENTHIRAN SREETHARAN, H. B. Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University

MASTER OF SCIENCE (2017) McMaster University

(Biology) Hamilton, Ontario

TITLE: Prenatal ionizing radiation exposure effects on cardiovascular health and disease in C57Bl mice

AUTHOR: Shayenthiran Sreetharan, H. B. Sc.

SUPERVISOR: Dr. Joanna Y. Wilson

NUMBER OF PAGES: ix, 79

LAY ABSTRACT

Women may be exposed to low dose radiation during pregnancy, particularly via diagnostic imaging, which raises concerns of harmful effects to the unborn baby. Exposure to stress (such as radiation) during pregnancy has been shown to lead to potentially life-long changes in the baby following birth, known as developmental programming. We used a mouse model to study the effects of radiation exposure during pregnancy on the cardiovascular system of the offspring. Higher doses of radiation decreased body size, increased blood pressure and decreased heart rate in female offspring. Necessary transportation of pregnant mice to the irradiation facility resulted in unintended effects on offspring, likely due to maternal stress, which was an unexpected result. This study is the first to investigate the effects of radiation exposure prior to birth on the cardiovascular system of offspring.

ABSTRACT

Ionizing radiation exposure during pregnancy raises concerns of potentially harmful effects for both the mother and the unborn child. Fetal programming involves permanent changes in offspring phenotype due to stress experienced *in-utero*. This phenomenon has been well characterized in cardiometabolic disorders such as hypertension. The effects of prenatal ionizing radiation exposure on offspring cardiovascular endpoints following birth were studied in a mouse model. Pregnant wildtype C57Bl/6J mice were irradiated on day 15 of pregnancy with whole-body ^{137}Cs gamma radiation at nominal doses of 5, 10, 50, 100, 300 or 1000 mGy. Post-natal measurements of offspring weight and blood pressure were completed. In female pups, blood pressure was significantly increased at 300 mGy and heart rate significantly decreased at 1000 mGy. Female pups were growth restricted over the study period at 50, 100 and 1000 mGy. Growth restriction in male pups was only observed at the highest dose of 1000 mGy. Unintended effects on the study measures caused by transportation of pregnant mothers to the irradiation facility were most evident in male offspring with increased blood pressure and heart rate and decreased body size. These unintended effects caused by transportation may have been attenuated with the 10 mGy *in-utero* exposure. Overall, these results suggest that prenatal radiation effects in mice are both dose- and gender-dependent, with even fairly low doses demonstrating (potentially adaptive) effects. There is a need for further study to better characterize the mechanism of this response.

ACKNOWLEDGEMENTS

I would like to thank Joanna Wilson for providing me the opportunity to pursue this research and for providing me constant guidance and mentorship as my supervisor over the course of this degree. I would also like to thank Doug Boreham and T. C. Tai at the Northern Ontario School of Medicine for their mentorship and support as well. I would like to thank all the members of the Wilson and Boreham Labs, past and present, that I've had the pleasure of meeting. There are far too many to list, but a particular thanks to Adam, Michael Lim, Lisa and Mary Ellen for company during our frequent visits to the library. Graduate school extends beyond the lab bench, and I was fortunate to have worked alongside an incredible group of students, technicians and post-doctoral fellows.

I am also grateful to Lisa Stoa and Mary Ellen Cybulski for their tireless technical support and guidance. Coming into this project without any prior mammal experience, their assistance for such a large, collaborative project made this possible. I'd also like to thank Devon Jones, Abigail Lee and Lisa Stoa for their assistance with the very labour intensive process of blood pressure measurements. Thank you to Grant McClelland for his contributions on my supervisory committee. I would like to acknowledge Bruce Power and NSERC for funding this research. Lastly, to my family and friends, thank you for the constant support and encouragement, without which I would not have been able to complete this thesis.

CONTRIBUTIONS

The material in Chapter 1 is published material in the Journal of Radiation Research. Copyright for inclusion of the article in this thesis was provided by the journal. Shayenthiran Sreetharan is first listed author of this article, providing the largest contribution and writing of the article. Christopher Thome, Sujeenthar Tharmalingam, Devon E. Jones, Adomas V. Kulesza, Neelam Khaper, Simon J. Lees, Joanna Y. Wilson, Douglas R. Boreham and T.C. Tai provided co-author contributions on the article. Individual co-author contributions are listed in Chapter 1.

The planning and experimental work in Chapter 2 was performed by Shayenthiran Sreetharan under the supervision of Dr. Joanna Wilson. Assistance with experimental work was provided by Lisa Stoa, Mary Ellen Cybulski and Adomas Kulesza.

Chapters 2 and 3 were prepared by Shayenthiran Sreetharan and edited by Dr. Joanna Wilson, Caitlin West, Allison Kennedy, Lisa Stoa and Adomas Kulesza.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA: Analysis of variance

ANCOVA: Analysis of co-variance

BP: Blood pressure

bpm: Beats per minute

Cort: Corticosterone

DBP: Diastolic blood pressure

GC: Glucocorticoid

Gy: Gray (unit of absorbed dose)

HR: Heart rate

MAP: Mean arterial pressure

ROS: Reactive oxygen species

SBP: Systolic blood pressure

SEM: Standard error of the mean

TLD: Thermoluminescent dosimeter

Chapter 1

General Introduction

Shayenthiran Sreetharan, Christopher Thome, Sujeenthar Tharmalingam, Devon E. Jones, Adomas V. Kulesza, Neelam Khaper, Simon J. Lees, Joanna Y. Wilson, Douglas R. Boreham and T. C. Tai

1.1 Copy-right declaration and co-author contributions

The content of chapter 1 is published material in the Radiation Research journal (doi: 10.1667/RR14657.1). Permission and nonexclusive license to McMaster University to reproduce this material as a part of this thesis was provided, with documentation supplied to the School of Graduate Studies at McMaster University. Text and tables have been formatted (appropriate heading numbering and spacing) compared to published version to conform to the formatting of this thesis. References are in order of appearance within the article, in non-alphabetical order.

The full citation of the article is below:

> **Ionizing Radiation Exposure During Pregnancy: Effects on Postnatal Development and Life**
>
> Shayenthiran Sreetharan, Christopher Thome, Sujeenthar Tharmalingam, Devon E. Jones, Adomas V. Kulesza, Neelam Khaper, Simon J. Lees, Joanna Y. Wilson, Douglas R. Boreham, and T. C. Tai
>
> *Radiation Research* **2017** 187 (6), 647-658

Co-authors to the article have also approved inclusion of the article and have acknowledged the contributions listed below:

- Christopher Thome: editing of manuscript, contribution towards section "Prenatal exposure to ionizing radiation in humans" (specifically low dose literature in humans), contribution towards organization of the paper

- Sujeenthar Tharmalingam: editing of manuscript, contribution towards introduction and "Animal studies"
- Devon E. Jones: editing of manuscript, contribution towards section "Prenatal exposure to ionizing radiation in humans" (specifically high dose literature in humans)
- Adomas V. Kulesza: editing of manuscript, contribution towards introduction
- Neelam Khaper, Simon J. Lees, Joanna Y. Wilson, Douglas R. Boreham & T. C. Tai: editing of the manuscript, overall literature review design and organization of the paper, assistance with funding

1.2 Abstract

[Sreetharan S, Thome C, Tharmalingam S, Jones DE, Kulesza AV, Khaper N, Lees SJ, Wilson JY, Boreham DR and Tai TC. Ionizing Radiation Exposure During Pregnancy: Effects on Postnatal Development and Life. *Radiation Research* 2017. 187 (6): 647-658]

Reliable human data on the effects of prenatal exposure to ionizing radiation is largely based on high-dose exposures. Exposure to low-doses may produce effects that are not easily observable at birth, and may persist over the course of the offspring's postnatal life. This is important when considering fetal programming, a phenomenon which is characterized by changes in offspring phenotype due to a stress experienced *in-utero*. In this review, we briefly summarize the known effects of ionizing radiation exposure during pregnancy in humans for both high-dose exposures and low-dose exposures. The major consensus regarding atomic bomb survivor data are increases in the incidence of microcephaly as well as IQ reductions where as diagnostic radiography *in-utero* does not have conclusive evidence of increased cancer risk. Due to the relatively limited data (particularly in low-dose exposures) in humans, animal models have emerged as an important tool to study prenatal effects due to the ability to manipulate various experimental parameters and the possibility to analyze a wider variety of endpoints. We discuss the major findings of studies from mouse and rat models examining prenatal ionizing radiation effects in postnatal development of the offspring. We also broadly categorize trends across studies within three major stages of development: pre-

implantation, organogenesis, and fetal development. Overall, long-term effects of prenatal radiation exposure (including the possible role on the developmental programming of disease) are important factors to consider when assessing radiation exposure risk as these effects are of relevance even in the low-dose range.

1.3 Introduction

Radiation exposure during pregnancy is a twofold problem; the concern for potential health effects to the mother and the unborn child. *In-utero* exposures to ionizing radiation can be quite diverse ranging from low-dose/dose-rate exposures (such as diagnostic radiography) to very high-dose/dose-rate exposures (such as the bombings of Hiroshima and Nagasaki). Other factors including the timing of exposure (with respect to gestational age of the developing offspring), type of radiation, and route of exposure all result in complex factors that must be taken into consideration when assessing risk.

An emerging area of concern is the potential for long-term effects on offspring due to prenatal radiation exposure. There is considerable evidence supporting the concept of developmental programming; the change in offspring phenotype or body processes due to an insult experienced *in-utero* (*1*, *2*). Although a variety of triggers have been identified, such as the exposure of an external stressor to the mother (i.e. hypoxia) or poor intra-uterine environment (i.e. due to malnutrition), the concept of developmental programming centers around the mother experiencing stress, and the developing fetus attempting to adapt to the stress due to transfer across the placenta *(2*, *3*).

With radiation known to be mutagenic and carcinogenic at high doses, there is a great deal of concern placed on limiting radiation exposure to pregnant females unless necessary. Available data on effects of ionizing radiation exposure in humans during prenatal development is largely based on the atomic bombings of Hiroshima and Nagasaki. These high-dose exposures serve as the basis for human protection standards, with effects extrapolated from high dose exposures; although this often does not reflect effects experienced in the low-dose range (*4*). Some of the more difficult effects to assess are sub-lethal endpoints such as behavioural or neurological effects which may persist into adulthood, but may not be present or are very difficult to identify at birth (*5*). Ionizing radiation (particularly in the low-dose range) may result in such effects, and is an area that is important to consider when assessing risk during pregnancy.

This review will summarize the available literature on the effects of ionizing radiation exposure *in-utero* and potential implications for long-term impacts on the offspring into adulthood. We briefly summarize the known effects of *in-utero* exposures to ionizing radiation in humans, covering both low-dose exposures (namely diagnostic radiography and nuclear medicine) and available high-dose exposures studies. We provide an overview of the available literature on effects observed in rodent species (mouse and rat), as a basis for investigation into the mechanistic and long-term effects of radiation exposure during pregnancy. We focus on studies that examined endpoints and effects at various times of post-natal life of the offspring. These findings are organized by timing of irradiation, during three major periods of development.

The term "low-dose" can be used in a variety of contexts, and for the purpose of this review we define low-dose to be doses that are ≤0.1 Gy (or 100 mGy) unless otherwise stated. The National Radiological Protection Board previously defined a low-dose as an acute dose of <100 mGy, or a dose rate of <5 mGy per hour (*6*). Although this generalization is being made, in real-world clinical scenarios, a number of factors must be considered when assessing risk including dose rate, radiation type and energy, affected tissues and any associated weighting factors, dosing schedule (i.e. a single, fractionated or protracted dosing regimen) and any other individual characteristics of the patient (such as age and gender).

1.4 Prenatal Exposure to Ionizing Radiation in Humans

One of the most common uses of ionizing radiation is in the field of medical radiography and nuclear medicine. Rough estimates of fetal absorbed doses during various treatments are summarized (Table I). Although there are reports of rough estimates for various diagnostic procedures, there are a number of factors that should be taken into consideration such as the timing of irradiation in pregnancy. For example, Winer-Muram et al. (*7*), reported a greater average fetal absorbed dose of helical computed tomography scans in the third trimester relative to the second trimester.

The dose delivered to the fetus from the majority of diagnostic nuclear medicine procedure exposures is often well below the threshold dose for deterministic effects (*8*). Therefore, the major area of perceived risk from prenatal diagnostic radiation exposure is excess cancer risk. Since the 1950's, a large number of case-control and cohort studies

have been conducted on *in-utero* exposure from diagnostic imaging procedures. The results of these studies are inconclusive as to whether prenatal exposure results in increased cancer risk. These studies have been previously reviewed by Boice and Miller (*9*), Brent (*10*), and Wakeford and Little (*11*).

Available data on prenatal radiation exposure to high doses is predominantly from studies on the survivors of the Hiroshima and Nagasaki atomic bombings who were pregnant at the time of exposure. The greatest consensus regarding effects from prenatal exposures at these high doses are reports of microcephaly and mental impairments. Microcephaly, defined as a head circumference of 2 or more standard deviations below the average for a given age and gender, was observed in 62 children exposed *in-utero* to the Hiroshima atomic bomb (4.21% of a total of 1473 that were irradiated prenatally and whose head circumference was measured between 9 and 19 weeks of age (*12*). A greater proportion of children presenting with microcephaly were irradiated in the first (55%) or second (31%) trimester with Miller (*13*) reporting only a single case of microcephaly later in gestation (between 26 and 40 weeks of gestation at a distance of 1201-1500 m from the hypocenter) which may suggest a more resistant period later in development (*12*). There does not appear to be a clear association between reduced head size and reports of reduced IQ scores. The mean IQ scores of cases who were assessed and clinically identified to be cognitively impaired with or without microcephaly was 63.8 and 68.9 respectively, with no significant difference between these two groups. Both groups assessed with cognitive impairments (with or without additional microcephaly) had significantly lower IQ scores compared to cases of children with reduced head size but

not assessed with cognitive impairment (*12*). Garsi et al. (*14*) reported no significant increases in the incidences of stillbirth, preterm births, low birth weight, congenital malformations and neonatal death during the first year of the offspring's life in women treated with ^{131}I radioiodine therapy. This was reported even in rare cases in which the total dose delivered to the ovaries can be on the scale of 140 to upwards of 1000 mGy.

1.5 Animal Studies

Due to limited data on radiation exposures during pregnancy in humans, this review will focus on the effects of radiation in pregnant rodent models. Rodent models allow precise manipulation of factors including radiation dose, radiation type, dose rate, and gestational age of exposure. In addition, a shorter life span for rodent species relative to humans more readily allows for investigation into long-term effects of radiation exposure *in-utero*. Two common rodent models, mice and rats were considered for this review. Prenatal ionizing radiation exposure studies are also available in other species such as dogs (*15*) and monkeys (*16*).

The overall irradiation factors (such as dose, dose rate, timing of exposure) are summarized in Tables II and III organized by major developmental periods of organogenesis (Table II) and fetal development (Table III). Major findings have been categorized based on three major stages of development: pre-implantation, organogenesis and the fetal period. In humans, the blastocyst has been reported to attach to the uterine wall by the sixth post-ovulatory day, with organogenesis occurring between the second to the end of 8 weeks gestation and fetal development for the last 30 weeks of gestation (*17*,

18). Pre-implantation in mice occurs for the first 5 days of gestation, organogenesis between days 8 and 15 of gestation, and fetal development between 14 and 20 days of gestation (*19*, *20*). Pre-implantation in rats has been reported to occur for the first 7 days of gestation, organogenesis between 8 and 15 days of gestation, and fetal development between 16 and 22 days of gestation (*19*, *20*).

1.5.1 Pre-implantation

Radiation exposure during the pre-implantation of the offspring is thought to have an "all-or-none" type response based on either complete lethality at higher doses, or no observable effect with lower doses (*21*). The all-or-none response was described by Russell & Russell (*22*), who studied pre-implantation irradiations using doses of 1-4 Gy and observed either lethal or null effects. The International Commission on Radiological Protection (ICRP) similarly published that the dominant effect of radiation exposure during pre-implantation is death and that the risk of malformation or induction of cancer to the offspring is unlikely (*23*, *24*). The lack of observable effects at low doses is due to the presence of totipotent cells within the developing offspring, which may allow for the regeneration of any cells that are irreversibly damaged by radiation exposure (*25-27*).

Contradictory evidence has emerged regarding this all-or-none response (*28-30*). There are reports of induced malformation caused by irradiation during the pre-implantation stage. For example, Streffer & Müller (*31*) reported increased malformation frequency from irradiation as early as the zygote stage, but also at subsequent pre-implantation stages. However, effects varied based on the mouse strain being studied.

A review by Adam (*32*) addresses the issue of the all-or-none phenomenon with respect to ionizing radiation exposure during the pre-implantation stage. It was concluded that the lack of evidence for radiation-induced pre-implantation malformations should not be interpreted as the inability of ionizing radiation exposure to cause lethality of the offspring, since malformations at that stage of development could be enough to cause lethality. This illustrates the need for better characterization of effects in the low-dose range. Although the all-or-none response may be valid at higher doses, it may not be the case at low doses. There may also be implications for effects later in life in the offspring other than effects that are observable prenatally or at birth.

1.5.2 Organogenesis

The effects reported in mouse and rat models during organogenesis and fetal development are more diverse than mortality of the offspring. The timing of the appearance of physiological markers of development have been used as a measure of effects on the physiology of the developing organism. These markers include fur development, pinna detachment, vaginal opening, descent of the testes, and eye opening. The threshold for effects on physiological markers during organogenesis appears to be between 0.2 and 0.4 Gy based on the results of Jensh & Brent (*33*) in Wistar rats who reported effects at 0.4 Gy 250 kVp x-ray irradiation but not at 0.2 Gy. Other studies have also reported radiation-induced effects on physiological markers during organogenesis, although these studies only tested (and reported effects at) doses of greater than 0.5 Gy.

Effects on the central nervous system appear to be diverse, including impacts on hippocampal structure and function, changes to the corpus callosum, astrocyte proliferation, and neuronal death (Table II). It was suggested these were due to the active development of the brain during gestational days 13-18 in mice (*34*). A number of conflicting studies investigated prenatal irradiation during organogenesis on post-natal behaviour (Table II). The threshold for behavioural effects appears to be around 0.10 Gy based on the results of Wang & Zhou (*35*) in C57Bl/6J mice who reported effects at 0.10 and 0.30 Gy β-radiation from tritiated water. Jensh et al. (*36*) however, did not see effects on behavioural testing in adulthood in Wistar rats irradiated with 250 kVp x-rays at doses of 0.10 and 0.20 Gy. Another area of uncertainty is whether behavioural changes are persistent into adulthood of the irradiated offspring. Minamisawa & Hirokaga (*37*) reported behavioural changes in C57Bl/6 x C3H mice in early (6-7 months) and mid (12-13 months) adulthood following exposure as low as 0.10 Gy ^{137}Cs γ-radiation. However, these reported effects were not present in late adulthood (19-20 months). Interestingly, at higher doses of 0.50 or 1.0 Gy (using the same study conditions), the effects were only observed in late adulthood (*38*). This may suggest that behavioural effects may diminish with time at lower doses, but effects persist into late adulthood if the dose is sufficiently high.

Similar to human reports, there are numerous reports of low birth weight and/or growth reduction in prenatally irradiated mice and rats during organogenesis (Table II). This is of particular relevance as low birth weight is a hallmark of developmental programming as a form of adaptation to stress experienced during *in-utero* development

(*39*). The majority of studies summarized in Tables I and II report low birth weight or growth retardation at higher doses (>0.5 Gy). Interestingly, Hande et al. (*40*) reported similar effects using 50 mGy 70 kVp x-ray irradiation but not at 9 mGy. The idea of x-irradiation requiring a lower threshold (between 9 and 50 mGy) for offspring growth effects is also supported by the results of Jensh et al. (*36*), who reported a threshold of 100-200 mGy in Wistar rats. Aside from differences in radiation type and quality, there is a lack of available literature on studies that investigate effects in the lower dose range (<0.10 Gy).

1.5.3 Fetal Development

Similar to organogenesis, the endpoints studied in animal models irradiated during fetal development (second and third trimester) are more diverse in comparison to pre-implantation. The threshold dose for impairments in learning and memory appears to be in the range of 0.25-0.30 Gy across the fetal period (*41-43*). Hossain & Uma Devi (*41*) reported impairments in learning and memory at doses of 0.50 Gy or greater using ^{60}Co γ-radiation in the early fetal period (day 14 in Swiss Albino mice) but not when irradiated with 0.25 Gy. Later in development at day 17 days, Uma Devi et al. (*42*) reported impairments with doses of 0.3 Gy or greater ^{60}Co γ-radiation. This was comparable to the reported results of Sienkiewicz et al. (*43*) who saw impairments in learning and memory testing in CD1 mice irradiated at day 18 with 0.35 or 0.50 Gy of 250 kVp x-rays, but had non-significant effects at lower doses of 0.10 or 0.25 Gy.

A variety of endpoints relating to central nervous system effects were studied during the fetal period but these studies were in the higher dose range of >1.0 Gy (Table III). Studies on the appearance of physiological markers of development also indicate a threshold below 0.30 Gy, which is comparable to effects during organogenesis (*44*). Physiological effects in Wistar rats at a higher dose of 2 Gy of 250 kVp x-rays decreased attainment of certain reflexes and changes in the appearance of four physiological markers of development: pinna detachment, eye opening, testis descent, and vaginal opening (*45*).

Irradiation during fetal development in mice and rats has also resulted in reports of low birth and/or growth retardation in offspring. At a very high dose (2 Gy of 250 kVp x-rays), Jensh et al. (*45*) reported persistent growth retardation and microcephaly in Wistar rats. Similarly, Uma Devi et al. (*42*) reported decreased body weight at doses of 0.30, 0.50, 1.0 and 1.5 Gy ^{60}Co γ-radiation. This reduced body weight was only significant for one week after birth at lower doses (0.3 and 0.5 Gy), however at the higher doses (1.0 and 1.5 Gy), this body weight reduction remained until the end of the study (6 months). These studies suggest that at high doses, programming of body size reduction appears to be persistent into adulthood of the animal.

Martin (46) also reported decreased size of liver, thymus, kidney, and spleen at birth due to decreased cell number and total DNA content in Sprague Dawley rats irradiated during the fetal stage (day 18) with 1.53 Gy of ^{60}Co γ-radiation. At 7 days post-irradiation, the thymus and spleen of irradiated animals grew at a faster rate than un-irradiated rats. Gui et al. (*47*) reviewed the topic of early programming of thymus size and

proposed the pre-natal and early post-natal periods of life to be critical periods for thymus development. Gamma irradiation was previously implicated in affecting thymus size and growth rate, increasing steady-state capacity of thymopoeisis in mice (*48*). Unlike the results of Martin (*46*) who reported decreased thymus size, Gui et al. reported larger thymus size 7 and 15 days post-irradiation compared to sham-irradiated controls. The thymus seems to be a potential target for investigating prenatal programming caused by radiation exposure,

Overall, these studies demonstrate the need to not only monitor size of the irradiated animal and organs at birth, and post-natal growth rate. This is particularly relevant when considering the "thrifty" or "catch-up" hypotheses of developmental programming of disease. Seckl and Holmes (*39*) and others have reported that a fetus undergoing programming decreases its overall metabolic capacity *in-utero* as an adaptation response to a stress. Following birth however, in the absence of such a stress, the animal may increase metabolism as a compensatory response, which may be observed as increased growth rate.

1.6 Conclusion

Exposure to low doses of ionizing radiation during pregnancy is often a concern due to the known detrimental effects at high doses. The majority of medical exposures in humans are low-dose exposures, and their effects, particularly related to developmental programming and other long-term changes in offspring phenotype, are important to consider when assessing risk to the offspring. The available literature does not provide

direct evidence that low-dose exposures results in increased stochastic effects (namely excess cancer risk) or deterministic effects to the offspring. Therefore, prenatal low-dose radiation exposure may not pose a risk to the developing offspring.

Radiation exposure during the pre-implantation stage of development in rodent models appear to follow an "all-or-none" response, based on complete lethality at higher doses or no visible effects. There is evidence that indicates this may not be true at lower doses. Exposures during organogenesis and fetal development have shown behavioural changes, impairments in learning and memory, effects on the central nervous system (which play an important role in elucidating potential mechanisms on behaviour, memory and learning), delay in the appearance of physiological markers of development, low birth weight and growth reduction. Reports of behavioural changes from irradiation during organogenesis are conflicting and it is unclear if these behavioural changes persist later into life. Effects on physiological markers of development during organogenesis and fetal development may have a threshold dose around 0.40 Gy. A threshold dose for effects on learning and memory during fetal development appears to be below 0.30 Gy, with reports of effects showing doses >0.30 Gy both during early and late fetal development. Low birth weight and growth retardation, both particularly important endpoints of interest due to their relevance and known programming effects on offspring weight, were reported both during organogenesis and fetal development. It appears that growth restriction may be transient at doses less than 1.0 Gy and permanent at higher doses (≥1.0 Gy). If the assumption is made that this growth reduction is evidence of programming, this illustrates the importance of studying other effects, which may be possible in the lower dose range.

Animal models such as the mouse and rat provide the opportunity to study endpoints not possible in human studies. Similar to the reported effects from atomic bomb survivors exposed *in-utero,* there were a number of reports in rodent models of impairments of the central nervous system. There is evidence of microcephaly in humans exposed to high acute doses prenatally, which has also been reported in animals. Uma Devi et al. (*49*) reported decreased brain weight and head size in mice irradiated at day 11.5 of gestation with doses >0.15 Gy. This study and others have reported effects on reduction in body and organ weight and overall growth reduction in prenatally irradiated animals. Reduction in body size is a hallmark of developmental programming of cardiovascular disease progression. Therefore, the role of prenatal ionizing radiation exposure (including low-dose exposures) on cardiovascular health of the offspring is an important area of future research.

1.7 Acknowledgements

The authors thank Dr. Charles Mitz and Michael Lim for critical review of the manuscript. The authors also thank the International Commission on Radiological Protection (ICRP) for the inclusion of Table I with permission. Funding was provided by Bruce Power. SS was supported by the Natural Sciences and Engineering Research Council of Canada's Canada Graduate Scholarship program. CT and ST were supported by a MITACS Accelerate grant.

1.8 Conflicts of Interest

None.

1.9 References

1. Barker, DJ, Gluckman, PD, Godfrey, KM, *et al.* Fetal nutrition and cardiovascular disease in adult life. *Lancet (London, England)*. 1993; **341**(8850): 938-41.

2. Reynolds, LP, Borowicz, PP, Caton, JS, *et al.* Developmental programming: The concept, large animal models, and the key role of uteroplacental vascular development. *Journal of Animal Science*. 2010; **88**(13 elect suppl): E61-E72.

3. Mulder, EJH, Robles de Medina, PG, Huizink, AC, *et al.* Prenatal maternal stress: effects on pregnancy and the (unborn) child. *Early Human Development*. 2002; **70**(1-2): 3-14.

4. Cohen, BL. The Cancer Risk from Low Level Radiation. In: Tack, D, Kalra, MK, Gevenois, PA (eds.). *Radiation Dose from Multidetector CT*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. 61-79.

5. Bilbo, SD, Schwarz, JM. The immune system and developmental programming of brain and behavior. *Frontiers in Neuroendocrinology*. 2012; **33**(3): 267-86.

6. Wakeford, R, Tawn, EJ. The meaning of low dose and low dose-rate. *Journal of Radiological Protection*. 2010; **30**(1): 1-3.

7. Winer-Muram, HT, Boone, JM, Brown, HL, *et al.* Pulmonary Embolism in Pregnant Patients: Fetal Radiation Dose with Helical CT1. *Radiology*. 2002; **224**(2): 487-92.

8. Steenvoorde, P, Pauwels, EKJ, Harding, LK, *et al.* Diagnostic nuclear medicine and risk for the fetus. *European Journal of Nuclear Medicine and Molecular Imaging*. 1998; **25**(2): 193-9.

9. Boice, JD, Jr., Miller, RW. Childhood and adult cancer after intrauterine exposure to ionizing radiation. *Teratology*. 1999; **59**(4): 227-33.

10. Brent, RL. Protection of the gametes embryo/fetus from prenatal radiation exposure. *Health physics*. 2015; **108**(2): 242-74.

11. Wakeford, R, Little, MP. Risk coefficients for childhood cancer after intrauterine irradiation: a review. *International Journal of Radiation Biology*. 2003; **79**(5): 293-309.

12. Otake, M, Schull, WJ. Radiation-related Small Head Sizes among Prenatally Exposed A-bomb Survivors. *International Journal of Radiation Biology*. 1993; **63**(2): 255-70.

13. Miller, RW. Delayed effects occurring within the first decade after exposure of young individuals to the Hiroshima atomic bomb. *Pediatrics*. 1956; **18**(1): 1-18.

14. Garsi, J-P, Schlumberger, M, Rubino, C, *et al.* Therapeutic Administration of 131I for Differentiated Thyroid Cancer: Radiation Dose to Ovaries and Outcome of Pregnancies. *Journal of Nuclear Medicine*. 2008; **49**: 845-52.

15. Benjamin, SA, Saunders, WJ, Angleton, GM, *et al.* Radiation Carcinogenesis in Dogs Irradiated During Prenatal and Postnatal Development. *Journal of Radiation Research*. 1991; **32**: 86-103.

16. Brizzee, KR, Ordy, JM, Kaack, MB, *et al.* Effect of prenatal ionizing radiation on the visual cortex and hippocampus of newborn squirrel monkeys. *Journal of Neuropathology and Experimental Neurology*. 1980; **39**(5): 523-40.

17. Strandring, S. *Gray's anatomy: the anatomical basis of clinical practice*. New York: Elsevier Limited; 2016.

18. Seeley, RR, Tate, P, Stephens, TD. *Anatomy & physiology*. Dubuque, IA: McGraw-Hill; 2008.

19. Streffer, C, Molls, M. Cultures of preimplantation mouse embryos: a model for radiobiological studies. 1987; **13**: 169-213.

20. UNSCEAR. Sources and effects of ionizing radiation: 1977 report to the General Assembly, with annexes. 1977.

21. Jacquet, P. Sensitivity of germ cells and embryos to ionizing radiation. *Journal of Biological Regulators and Homeostatic Agents*. 2004; **18**(2): 106-14.

22. Russell, LB, Russell, WL. *The effects of radiation on the preimplantation stages of the mouse embryo*: John Wiley & Sons Inc.; 1950.

23. ICRP. Recommendations of the International Commission on Radiological Protection. ICRP Publication 60. 1991; **21**(1-3).

24. Valentin, J. *Biological effects after prenatal irradiation (embryo and fetus)*. Oxford: Pergamon; 2003.

25. Brent, RL. Utilization of developmental basic science principles in the evaluation of reproductive risks from pre- and postconception environmental radiation exposures. *Teratology*. 1999; **59**(4): 182-204.

26. Moore, NW, Adams, CE, Rowson, LEA. Developmental potential of single blastomeres of the rabbit egg. *Reproduction*. 1968; **17**(3): 527-31.

27. Willadsen, SM. A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature*. 1979; **277**(5694): 298-300.

28. Gu, Y, Kai, M, Kusama, T. The embryonic and fetal effects in ICR mice irradiated in the various stages of the preimplantation period. *Radiation Research*. 1997; **147**(6): 735-40.

29. Gu, YH, Hasegawa, T, Suzuki, I. Combined effects of radiation and ultrasound on ICR mice in the preimplantation stage. *Ultrasound in Medicine & Biology*. 2002; **28**(6): 831-6.

30. Pampfer, S, Streffer, C. Prenatal death and malformations after irradiation of mouse zygotes with neutrons or X-rays. *Teratology*. 1988; **37**(6): 599-607.

31. Streffer, C, Müller, WU. Malformations after radiation exposure of preimplantation stages. *The International Journal of Developmental Biology*. 1996; **40**(1): 355-60.

32. Adam, MP. The all-or-none phenomenon revisited. *Birth Defects Research Part A: Clinical and Molecular Teratology*. 2012; **94**(8): 664-9.

33. Jensh, RP, Brent, RL. The Effects of Prenatal X Irradiation on the Appearance of Reflexes and Physiologic Markers in the Neonatal Rat. *Radiation Research*. 1988; **116**(3): 416.

34. Hossain, M, Chetana, M, Uma Devi, P. Late effect of prenatal irradiation on the hippocampal histology and brain weight in adult mice. *International Journal of Developmental Neuroscience*. 2005; **23**(4): 307-13.

35. Wang, B, Zhou, X-Y. Effects of Prenatal Exposure to Low-dose β Radiation from Tritiated Water on the Neurobehavior of Mice. *Journal of Radiation Research*. 1995; **36**(2): 103-11.

36. Jensh, RP, Brent, RL, Vogel, WH. Studies Concerning the Effects of Low Level Prenatal X-irradiation on Postnatal Growth and Adult Behaviour in the Wistar Rat. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine*. 1986; **50**(6): 1069-81.

37. Minamisawa, T, Hirokaga, K. Long-Term Changes in Open Field Activity of Male Mice Irradiated with Low Levels of Gamma Rays at Late Stage of Development. *Journal of Radiation Research*. 1996; **37**(2): 117-24.

38. Minamisawa, T, Hirokaga, K. Long-Term Effects of Prenatal Exposure to Low Levels of Gamma Rays on Open-Field Activity in Male Mice. *Radiation Research*. 1995; **144**(2): 237-40.

39. Seckl, JR, Holmes, MC. Mechanisms of Disease: glucocorticoids, their placental metabolism and fetal 'programming' of adult pathophysiology. *Nature Clinical Practice Endocrinology & Metabolism*. 2007; **3**(6): 479-88.

40. Hande, MP, Uma Devi, P, Jagetia, GC. Effect of "In Utero" Exposure to Low Doses of Low Energy X-rays on the Postnatal Development of Mouse. *Journal of Radiation Research*. 1990; **31**(4): 354-60.

41. Hossain, M, Uma Devi, P. Effect of irradiation at the early foetal stage on adult brain function of mouse: learning and memory. *International Journal of Radiation Biology*. 2001; **77**(5): 581-5.

42. Uma Devi, P. Effect of gamma radiation on the foetal haemopoietic system in the mouse. *International Journal of Radiation Biology*. 1999; **74**(5): 639-46.

43. Sienkiewicz, ZJ, Haylock, RGE, Saunders, RD. Prenatal Irradiation and Spatial Memory in Mice: Investigation of Dose-response Relationship. *International Journal of Radiation Biology*. 1994; **65**(5): 611-8.

44. Hossain, M, Uma Devi, P, Bisht, KS. Effect of prenatal gamma irradiation during the late fetal period on the postnatal development of the mouse. *Teratology*. 1999; **59**(3): 133-8.

45. Jensh, RP, Eisenman, LM, Brent, RL. Postnatal Neurophysiologic Effects of Prenatal X-irradiation. *International Journal of Radiation Biology*. 1995; **67**(2): 217-27.

46. Martin, PG. The Postnatal Response of Four Organs to Prenatal Irradiation as Measured by Changes in Nucleic Acids and Protein. *Radiation Research*. 1971; **48**(2): 368.

47. Gui, J, Mustachio, LM, Su, D-M, *et al.* Thymus Size and Age-related Thymic Involution: Early Programming, Sexual Dimorphism, Progenitors and Stroma. *Aging and Disease*. 2012; **3**(3): 280-90.

48. Gui, J, Morales, AJ, Maxey, SE, *et al.* MCL1 increases primitive thymocyte viability in female mice and promotes thymic expansion into adulthood. *International Immunology*. 2011; **23**(10): 647-59.

49. Uma Devi, P, Baskar, R, Hande, MP. Effect of Exposure to Low-Dose Gamma Radiation during Late Organogenesis in the Mouse Fetus. *Radiation Research*. 1994; **138**(1): 133.

50. ICRP. Pregnancy and Medical Radiation. ICRP Publication 84. *ICRP* 2000; **30**(1).

51. Rugh, R, Wohlfromm, M. Prenatal X-Irradiation and Postnatal Mortality. *Radiation Research*. 1965; **26**(4): 493.

52. Hande, MP, Uma Devi, P. Teratogenic effects of repeated exposures to X-rays and/or ultrasound in mice. *Neurotoxicology and Teratology*. 1995; **17**(2): 179-88.

53. Nash, DJ. Effects of Prenatal X-Irradiation on Development and Postnatal Viability of Inbred and Hybrid Mice. *The Biological Bulletin*. 1971; **140**(2): 230-41.

54. Nash, DJ, Gowen, JW. Effects of X-Irradiation Upon Postnatal Growth in the Mouse. *The Biological Bulletin*. 1962; **122**(1): 115-36.

55. Zhong, D. Neurobehavioral study of prenatal exposure to hyperthermia combined with irradiation in mice. *Neurotoxicology and Teratology*. 1996; **18**(6): 703-9.

56. Sikov, MR, Resta, CF, Lofstrom, JE. The Effects of Prenatal X-Irradiation of the Rat on Postnatal Growth and Mortality. *Radiation Research*. 1969; **40**(1): 133.

57. Wang, B, Ohyama, H, Haginoya, K, *et al.* Adaptive Response in Embryogenesis: II. Retardation of Postnatal Development of Prenatally Irradiated Mice. *Radiation Research*. 1999; **152**(2): 119-23.

58. Wang, B, Ohyama, H, Shang, Y, *et al.* Adaptive Response in Embryogenesis: V. Existence of Two Efficient Dose-Rate Ranges for 0.3 Gy of Priming Irradiation to Adapt Mouse Fetuses. *Radiation Research*. 2004; **161**(3): 264-72.

59. Kimler, BF, Norton, S. Behavioral changes and structural defects in rats irradiated in utero. *International Journal of Radiation Oncology*Biology*Physics*. 1988; **15**(5): 1171-7.

60. Baskar, R, Uma Devi, P. Influence of gestational age to low-level gamma irradiation on postnatal behavior in mice. *Neurotoxicology and Teratology*. 2000; **22**(4): 593-602.

61. Reyners, EGDRH. Delayed effects of prenatal low-dose irradiation in the white matter of the rat brain. *International Journal of Radiation Biology*. 1999; **75**(10): 1327-34.

62. Gao, W-M, Lu, H-M, Dong, J-C, *et al.* Postnatal growth, neurobehavioral and neurophysiologic changes of prenatal low-dose β-radiation from tritiated water in mice. *Neurotoxicology and teratology*. 2002; **24**(2): 247-54.

63. Korr, HTRJBH. Neuron loss during early adulthood following prenatal low-dose X-irradiation in the mouse brain. *International Journal of Radiation Biology*. 2001; **77**(5): 567-80.

64. Sienkiewicz, ZJ. Differential learning impairments produced by prenatal exposure to ionizing radiation in mice. *International Journal of Radiation Biology*. 1999; **75**(1): 121-7.

65. Sienkiewicz, ZJ, Saunders, RD, Butland, BK. Prenatal Irradiation and Spatial Memory in Mice: Investigation of Critical Period. *International Journal of Radiation Biology*. 1992; **62**(2): 211-9.

66. Janeczko, K, Pawliński, R, Setkowicz, Z, *et al*. Long-term postnatal effect of prenatal irradiation on the astrocyte proliferative response to brain injury. *Brain Research*. 1997; **770**(1–2): 237-41.

67. Janeczko, K, Setkowicz, Z, Frączek, M, *et al.* Effects of prenatal gamma-irradiation on postnatal astrogliogenesis in the hippocampal formation of rat. *Brain Research*. 1999; **816**(2): 628-32.

68. Murphree, RL, Pace, HB. The Effects of Prenatal Radiation on Postnatal Development in Rats. *Radiation Research*. 1960; **12**(5): 495.

69. Schmitz, C, Born, M, Dolezel, P, *et al.* Prenatal protracted irradiation at very low dose rate induces severe neuronal loss in rat hippocampus and cerebellum. *Neuroscience*. 2005; **130**(4): 935-48.

70. Li, H-P, Miki, T, Gu, H, *et al.* The effect of the timing of prenatal X-irradiation on Purkinje cell numbers in rat cerebellum. *Developmental Brain Research*. 2002; **139**(2): 159-66.

71. Norton, S. Behavioral changes in preweaning and adult rats exposed prenatally to low lonizing radiation. *Toxicology and Applied Pharmacology*. 1986; **83**(2): 240-9.

72. Kitamura, M, Itoh, K, Matsumoto, A, *et al.* Prenatal ionizing radiation-induced apoptosis of the developing murine brain with special references to the expression of some proteins. *Kobe Journal of Medical Sciences*. 2001; **47**(2): 59-76.

73. Minamisawa, T, Hirokaga, K, Sasaki, S. Gross Morphological Changes of the Mouse Brain Exposed Prenatally to Ionizing Radiation. *Journal of Radiation Research*. 1990; **31**(2): 214-8.

74. Minamisawa, T, Hirokaga, K. Long Term Effects of Prenatal Exposure to Low Level Gamma Rays on Spontaneous Circadian Motor Activity of Male Mice. *Journal of Radiation Research*. 1995; **36**(3): 179-84.

75. Hossain, M, Uma Devi, P. Effect of irradiation at the early fetal stage on adult brain function in the mouse: locomotor activity. *International Journal of Radiation Biology*. 2000; **76**(10): 1397-402.

76. Uma Devi, P, Hossain, M. Induction of chromosomal instability in mouse hemopoietic cells by fetal irradiation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. 2000; **456**(1–2): 33-7.

77. Hossain, P, Uma Devi, M. Induction of solid tumours in the Swiss albino mouse by low-dose foetal irradiation. *International journal of radiation biology*. 2000; **76**(1): 95-9.

78. Tomášová, L, Šmajda, B, Ševc, J. Effects of prenatal irradiation on behaviour and hippocampal neurogenesis in adult rats. *Acta Physiologica Hungarica*. 2012; **99**(2): 126-32.

79. Uma Devi, P, Hossain, M, Bisht, KS. Effect of late fetal irradiation on adult behavior of mouse: Dose-response relationship. *Neurotoxicology and Teratology*. 1999; **21**(2): 193-8.

80. Kokošová, N, Tomášová, L, Kisková, T, *et al.* Neuronal Analysis and Behaviour in Prenatally Gamma-Irradiated Rats. *Cellular and Molecular Neurobiology*. 2015; **35**(1): 45-55.

81. Werboff, J, Goodman, I, Havlena, J, *et al.* Effects of prenatal x-irradiation on motor performance in the rat. *American Journal of Physiology--Legacy Content*. 1961; **201**(4): 703-6.

82. Werboff, J, Havlena, J, Sikov, MR. Effects of Prenatal X-Irradiation on Activity, Emotionality, and Maze-Learning Ability in the Rat. *Radiation Research*. 1962; **16**(4): 441.

Table 1.1: Approximate absorbed dose to the fetus from various diagnostic procedures. Table reproduced with permission of the author (*50*).

Procedure	Mean (mGy)	Maximum (mGy)
Conventional x-ray examination		
Abdomen	1.4	4.2
Chest	<0.01	<0.01
Intravenous urogram	1.7	10
Lumbar spine	1.7	10
Pelvis	1.1	4
Skull	<0.01	<0.01
Thoracic spine	<0.01	<0.01
Fluoroscopic examination		
Barium meal (upper gastrointestinal)	1.1	5.8
Barium enema	6.8	24
Computed tomography (CT)		
Abdomen	8.0	49
Chest	0.06	0.96
Head	<0.005	<0.005
Lumbar spine	2.4	8.9
Pelvis	25	79

Table 1.2: Summary table of postnatal effects of prenatal irradiations during organogenesis in mice (gestational days 6-13) and rats (gestational days 8-15). Details of irradiation protocol including radiation type, doses administered and dose rate used are included where available. Day of irradiation and age of offspring at the time of testing provided.

	Radiation Type	Dose (Gy)[a]	Dose Rate (Gy/ min)	Day of Irradiation	Age of Offspring at Time of Testing[b]	Results[c,d,e,f]
Rugh & Wohlfromm (*51*)	184 kVp x-ray	1.10-5.35	0.44	Daily following conception (Mouse)	-30 days	-No effect on sex ratio -Most animals died within 48 hours of birth -Neonatal deaths frequently due to exencephaly, microcephaly, hydrocephaly, and anemia -Appendage abnormalities for E11/12 irradiations
Hande et al. (*40*)	70 kV x-ray	0.009, 0.05	0.9	6.5, 11.5 (Mouse)	-Up to 6 weeks	-LBW in early (E6.5) and late (E11.5) organogenesis -Threshold (50 mGy): post-natal death in early organogenesis
Hande & Uma Devi (*52*)	70 kVp x-ray; 3.5 MHz ultrasound	9mGy (x-ray), 10 minutes (ultrasound)	0.9	6.5 and 11.5 (Mouse)	-E18 -6 weeks -3 or 6 months	-Significant decrease in birth weight in all groups except ultrasound + x-ray until 4 weeks of age -No effects on organ/body weight ratios -Effects on LM and Behav. with x-ray + ultrasound or ultrasound-only treatment
Nash (*53*)	250 kVp x-ray	0.18, 0.70, 1.40, 2.81	1.17	6.5, 10.5, (Mouse)	-Up to 75 days	-Morphological anomalies at E10.5 (>0.80 Gy) -LD50birth between 0.70 and 1.40 Gy (highest at E10.5) -Lowest post-natal survival with E6.5 irradiation, highest with E10.5

Nash & Gowen (*54*)	250 kVp x-ray	0.18, 0.70, 1.40, 2.81	1.17	6.5, 10.5, (Mouse)	-Up to 75 days	-Persistent growth effects -Dose and gestational age of irradiation dependant
Zhong et al. (*55*)	^{60}Co γ-radiation	0.50	0.25	9 (Mouse)	-Up to 49 days	-LBW -Delay in Physiol. -No effect of hyperthermia or hyperthermia treatment with radiation
Jensh et al. (*36*)	250 kVp x-ray	0.10, 0.20	0.12-0.14	9 (Rat)	-60 days	-No effects on adult behaviour -LBW in females and males (in females the growth retardation persisted)
Jensh & Brent (*33*)	250 kVp x-ray	0.10, 0.20, 0.40, 0.60, 0.80	0.12-0.14	9 (Rat)	-Birth until the entire litter completed the reflex response or all physiological markers were observed	-Dose-dependent delay in eye opening -No effect on litter size
Sikov et al. (*56*)	250 kVp x-ray	0.18, 0.44, 0.88, 1.62	0.16	10, 15 (Rat)	-2, 4, 6 months -3 months apart thereafter	-Decreased birth weight in all treatments except E10/0.18 Gy -Growth retardation only with E15 irradiations -Greatest increase in mortality with E15/1.62 Gy treatment, greater mortality in female relative to male irradiations
Wang et al. (*57*)	Not indicated	0.30 priming, 5.00 challenge	Not indicated	11 (priming), 12 (challenge; Mouse)	-Up to 180 days	-Effects on Physiol. -Altered adult behaviour -Combined doses resulted in decreased body weight for both sexes -Priming dose was beneficial for offspring survival, but the offspring which survived had detrimental effects

Wang et al. (*58*)	200 kVp x-ray	0.30 priming, 3.50 challenge	0.06 - 5.0 (priming), 1.8 (challenge)	11 (priming), 12 (challenge; Mouse)	-Up to 30 days	-Priming dose significantly reduced prenatal deaths, postnatal mortality and malformation incidence (apoptosis in limb buds and digital defects) -Adaptive response was observed when irradiated at a dose rate of 0.18-0.98 or 3.5-4.6 Gy/min (same priming dose)
Kimler & Norton (*59*)	^{137}Cs γ-radiation	0.25, 0.50, 0.75, 1.00, 1.25	0.52	11, 13, 15 (Rat)	-28 days	-Dose response observed for behavioural effects E15 irradiation -One behavioural test significantly affected as low as 0.25 Gy -Significant reduction in body weight at 1.0 and 1.25 Gy, E15 irradiation -Anterior pituitary-based effects most sensitive at E11 -E15 irradiation sensitive for behavioural changes, morphological changes and effects on body weight
Baskar & Uma Devi (*60*)	^{60}Co γ-radiation	0.25, 0.35, 0.50	0.83	11.5, 12.5, (Mouse)	-3 months	-Organogenesis more sensitive than fetal period (E11.5 most sensitive) -Significant reduction in locomotor and exploratory activities at 0.35 Gy for E11.5 and 12.5 irradiations -Threshold (0.5 Gy) for Behav. for all tested gestational days
Reyners et al. (*61*)	250 kVp x-ray	0.18, 0.50	0.6	12, 13, 14, 15 (Rat)	-1, 3 months	-Atrophy (likely neuronal loss) in the cingulum of the corpus callosum, -Increased severity at 3 months with E15 irradiation
Gao et al. (*62*)	$^{3}H_2O$ β-radiation	0.036, 0.071, 0.213	Activity: 38.3 x 10^4 Bq/ul	12.5 (Mouse)	-Up to 56 days	-Dose response observed -Neuronal death & impairment with 0.071 and 0.213 Gy irradiation

Wang & Zhou (*35*)	$^{3}H_2O$ β-radiation	0.050, 0.10, 0.30	Activity not indicated	12.5 (Mouse)	-Up to 118 days	-Greater effect from chronic β-irradiation with respect to a comparable acute dose of x- or gamma irradiation -Threshold (10 cGy): LM effects
Korr et al. (*63*)	250 kV x-ray	0.10, 0.50	0.59	13 (Mouse)	-E14 -25, 180 days	-Cell death in adult F1's due to accumulated mitochondrial DNA damage
Sienkiewicz (*64*)	250 kV x-ray	1.00	0.73	13 (Mouse)	-21 to 24 weeks	-No significant effects on LM tasks with E13 irradiation
Sienkiewicz et al. (*65*)	250 kV x-ray	1.00	0.73	13 (Mouse)	-Up to 52 days	-Sustained deficits in acquisition of spatial information on E18 or post-natal day 1 -Effects not observed on E13 or E15
Janeczko et al. (*66*)	^{60}Co γ-radiation	1.00	1.53	13, 15 (Rat)	-31-34 days	-Brain histology: significant decrease in the thickness of the cerebral hemisphere wall and enlargement of the lateral venticles -Increase reactive astrocyte proliferation following E15 irradiation
Janeczko et al. (*67*)	^{60}Co γ-radiation	1.00	1.53	13, 15 (Rat)	- 2, 4, 8, 16 and 30 days	-Intensity of astrocyte proliferation decreased, especially in the gentate gyrus -Effects more pronounced in E19 irradiations compared to E13
Murphee & Pace (*68*)	^{60}Co γ-radiation	1.10, 1.50, 2.20	0.041	13-20 (Rat)	-Up to 90 days	-Increased neonatal deaths with E13-E18 irradiation -Majority of postnatal deaths occurred by day 7 -Lower average weight in all irradiated groups -Decreased ovulation in prenatally irradiated females

						-Decrease in mature body and testes weight of prenatally irradiated males, related to dose and gestational age of irradiation
Schmitz et al. (*69*)	^{60}Co γ-radiation	3.00 cumulative dose	7 x 10^{-4}	13 to 16 (continuous; Rat)	-Up to 30 months	-Decreased number of hippocampal pyramid and granule cells -No significant effect on neuron density -Effects variable compared to a single, acute high dose rate irradiation
Li et al. (*70*)	180 kV x-ray	1.50	0.23	14, 15, 16 (Rat)	-7 weeks	-E14 and E15 but not E16 irradiations resulted in decreased body weight, whole brain weight, cerebellar weight and Purkinje cell numbers
Norton (*71*)	250 kV x-ray	0.25, 0.50, 0.75, 1.25	0.40	15 (Rat)	-Up to 21 days	-Some behavioural tests showed dose-response relationship (as low as 0.25 Gy) while other tests had no effect -LBW in early life only -Delays in behavioural life, only in early life

[a] An approximate conversion of 1 R = 8.77 mGy was used for studies with dosing given in Roentgen
[b].E# indicates irradiation on the #th day of gestation
[c] LBW: low birth weight or growth retardation
[d] Physiol.: timing and onset of physiological landmarks of development
[e] Behav. : behavioural testing
[f] LM: learning and memory testing.

Table 1.3: Summary table of postnatal effects of prenatal irradiations during fetal development in mice (gestational days 14-20) and rats (gestational days 16-22). Details of irradiation protocol including radiation type, doses administered (in Gy unless otherwise stated) and dose rate (in Gy/min unless otherwise stated) used are included where available. Day of irradiation and age of offspring at the time of testing provided.

	Radiation Type	Dose (Gy)[a]	Dose Rate (Gy/ min)	Day of Irradiation	Age of Offspring at Time of Testing[b]	Results[c,d,e,f]
Rugh & Wohlfrom m (*51*)	184 kVp x-ray	1.10-5.35	0.44	Daily following conception	-30 days	-No effect on sex ratio -Most animals died within 48 hours of birth -Neonatal deaths frequently due to exencephaly, microcephaly, hydrocephaly, and anemia
Murphee & Pace (*68*)	^{60}Co γ-radiation	1.10, 1.50, 2.20	0.041	13-20 (Rat)	-Up to 90 days	-Increased neonatal deaths with E13-E18 irradiation -Majority of postnatal deaths occurred by day 7 -Lower average weight in all irradiated groups -Decreased ovulation in prenatally irradiated females -Decrease in mature body and testes weight of prenatally irradiated males, related to dose and gestational age of irradiation
Hossain & Uma Devi (*41*)	^{60}Co γ-radiation	0.25, 0.50, 1.00, 1.50	1.00	14 (Mouse)	-6, 12, 18 months	-Dose response observed for LM effects, linear up to 1 Gy -Defects in LM of 6 month mice, >0.25 Gy -These effects persisted to 18 months, at doses of 0.50 Gy and higher
Kitamura et al. (72)	150 kVp x-ray	1.50	1.65	14 (Mouse)	-E15, E17, E19 -1, 3, 5, 7, 10, 14, 21 days	-Extensive apoptosis observed at E15 and E17 -Microcephaly observed postnatally -Significantly decreased brain weight at post natal days 7 and 21

Minamisawa et al. (*73*)	^{137}Cs γ-radiation	1.00, 2.00, 3.00	Not indicated	14 (Mouse)	-6 months	-Irradiated pups were fostered by normal mothers -Significant, dose-dependent reduction in brain and body weight at all doses -Detrimental effects on the area of the superior colliculi -Threshold (<1 Gy) for effects studied
Minamisawa & Hirokaga (*38*)	^{137}Cs γ-radiation	0.50, 1.00	0.17	14 (Mouse)	-6-7 months -12-13 months -19-20 months	-LBW -Behavioural effects, only observed in late life (19-20 months)
Minamisawa and Hirokaga (*74*)	^{137}Cs γ-radiation	0.10, 0.20, 0.50, 1.00	0.17	14 (Mouse)	-6-7 months -12-13 months -19-20 months	-Nocturnal hyperactivity (young and adult males) -Threshold (1 Gy) for effects on circadian rhythm
Minamisawa and Hirokaga (*37*)	^{137}Cs γ-radiation	0.10, 0.20	0.17	14 (Mouse)	-6-7 months -12-13 months -19-20 months	-Behavioural effects observed 6-7 and 12-13 weeks -Effects were not persistent (19-20 months)
Hossain & Uma Devi (*75*)	^{60}Co γ-radiation	0.25, 0.30, 0.50, 1.00, 1.50	1.00	14.5 (Mouse)	-6, 12, 18 months	-Effects on behavioural testing -Threshold (0.3 Gy): Behav. -Linear dose response observed -Effects at 12 months, not persistent (18 months)
Hossain et al. (*34*)	^{60}Co γ-radiation	0.25, 0.50, 1.00, 1.50	1.00	14, 17 (Mouse)	-6, 12 months	-Threshold (0.5 Gy): brain weight changes -Threshold (1 Gy): decreased neuron number -Permanent changes in brain histology
Uma Devi & Hossain (*76*)	^{60}Co γ-radiation	0.25, 0.50, 1.00, 1.50	1.00	14, 17 (Mouse)	-12 months	-Low peripheral blood counts -Dose response observed -Aberrant metaphase chromosomes present in bone marrow

Hossain & Uma Devi (*77*)	Not indicated	0.10, 0.25, 0.30, 0.50, 1.00, 1.50	1.00	14, 17 (Mouse)	-6-18 months	-Linear quadratic dose response increase in tumour incidence with E14 and 17 irradiation -Highest tumour incidence in ovaries -Earlier formation of tumours of the ovaries and uterus than spleen or liver
Baskar & Uma Devi (*60*)	^{60}Co γ-radiation	0.25, 0.35, 0.50	0.83	14.5, 17.5 (Mouse)	-3 months	-Late fetal period relatively resistant (17.5 days) -Threshold (0.5 Gy) for Behav. for all tested gestational days
Nash & Gowen (*54*)	250 kVp x-ray	0.18, 0.70, 1.40, 2.81	1.17	14.5, 17.5 (Mouse)	-Up to 75 days	-Persistent growth effects -Dose and gestational age of irradiation dependant
Sienkiewicz (*64*)	250 kV x-ray	1.00	0.73	15, 18 (Mouse)	-21 to 24 weeks	-LM tasks affected with E15 and E18 irradiations
Sienkiewicz et al. (*65*)	250 kV x-ray	1.00	0.73	15, 18 (Mouse)	-Up to 52 days	-Sustained deficits in acquisition of spatial information on E18 or post-natal day 1 -Effects not observed on E13 or E15
Tomášová et al. (*78*)	^{60}Co γ-radiation	1.00	Not indicated	16 (Rat)	-3 months	-Negatively affected short- but not long-term memory in females -Increased anxiety behaviour in females -Increased basic locomotor activity -No effects on hippocampal neurogenesis
Uma Devi et al. (*79*)	^{60}Co γ-radiation	0.30, 0.50, 1.00, 1.50	Not indicated	17 (Mouse)	-6 months	-Linear dose response observed for behavioural testing -Linear dose response observed for brain weights - Threshold (0.3 Gy): LM
Hossain et al. (*44*)	^{60}Co γ-radiation	0.30, 0.50, 1.00, 1.50	Not indicated	17 (Mouse)	-6 weeks	-LBW for one week at all doses, persistent to 6 months at 1.0 and 1.5 Gy -Linear dose response observed for behavioural testing

						-Linear dose response observed for effects on Physiol. parameters -Fur development affected at 0.3 Gy, all other Physiol. parameters at doses >0.3 Gy
Jensh et al. (*36*)	250 kVp x-ray	0.10, 0.20	0.12-0.14	17 (Rat)	-60 days	-No effects on adult behaviour -LBW in females and males (in females the growth retardation persisted)
Jensh & Brent (*33*)	250 kVp x-ray	0.10, 0.20, 0.40, 0.60, 0.80	0.12-0.14	17 (Rat)	-Birth until the entire litter completed the reflex response or all physiological markers were observed	-Dose-dependent decrease in litter size, although not significant -Dose response observed for Physiol. changes -Threshold (0.2-0.4 Gy): Physiol. effects
Jensh et al. (*45*)	250 kVp x-ray	2.00	0.12-0.14	17 (Rat)	-Weekly up to 20 weeks	-Permanent growth retardation, microcephaly and alterations of the hippocampus -Decreased attainment of certain reflexes -Changes in timing of physiologic markers -Changes in adult behaviour
Kokošová et al. (*80*)	^{60}Co γ-radiation	1.00	Not indicated	17 (Rat)	-3 weeks -2, 3 months	-Decrease in the number of mitotically active cells in the gentate gyrus (males) -Decrease in mature neurons in CA1/gentate gyrus regions of hippocampus -Negative effects on LM, decreased exploratory behaviour, increased anxiety-like behaviour; all seen at 2 months but not 3 months
Janeczko et al. (*66*)	^{60}Co γ-radiation	1.00	1.53	17, 19 (Rat)	-31-34 days	-Brain histology: significant decrease in the thickness of the cerebral hemisphere wall and enlargement of the lateral ventricles

Janeczko et al. (*67*)	^{60}Co γ-radiation	1.00	1.53	17, 19 (Rat)	- 2, 4, 8, 16 and 30 days	-Intensity of astrocyte proliferation decreased, especially in the gentate gyrus -Effects more pronounced in E19 irradiations compared to E13
Werboff et al. (*81*)	250 kVp x-ray	0.25, 0.50, 1.00	0.094	20 (Rat)	-Up to 120 days	-Decreased motor strength at almost all doses -Effects at all studied doses
Werboff et al. (*82*)	250 kVp x-ray	0.25, 0.50, 1.00	0.094	20 (Rat)	-Up to 120 days	-Effects on activity, emotionality and LM -Differing effects in females based on gestational age of irradiation (not seen in males) -No clear dose response observed

[a] An approximate conversion of 1 R = 8.77 mGy was used for studies with dosing given in Roentgen.
[b] E# indicates irradiation on the #th day of gestation
[c] LBW: low birth weight or growth retardation
[d] Physiol.: timing and onset of physiological landmarks of development
[e] Behav. : behavioural testing
[f] LM: learning and memory testing.

Chapter 2

Fetal programming of C57Bl mice with physical transportation and adaptive response with prenatal low dose ionizing radiation exposure

Shayenthiran Sreetharan, Lisa Stoa, Mary Ellen Cybulski, Adomas V. Kulesza, Douglas R. Boreham, T.C. Tai and Joanna Y. Wilson

2.1 Abstract

Developmental programming is a phenomenon in which an adverse intrauterine environment can result in a change in offspring phenotype following birth. The developmental programming of hypertension and other cardiometabolic diseases has been implicated by maternal stress, with a possible mechanism of oxidative stress at the cellular level. Ionizing radiation produces oxidative stress, even at low doses, and irradiation of animals is often coupled with potential sources of maternal stress such as transportation to specialized irradiation facilities. Pregnant C57Bl/6J mice were irradiated on gestational day 15 with whole-body ^{137}Cs gamma radiation at nominal doses of 5, 10, 50, 100, 300 or 1000 mGy. Post-natal weight, blood pressure and heart rate were measured in offspring. Evidence of programming due to multiple transportations to the irradiation facility was evident in male pups. Sham-irradiated male pups had significantly elevated blood pressure and heart rate relative to naïve control pups from mothers that were not transported. Interestingly, 10 mGy attenuated these unintended effects in male pups, with a reduction in blood pressure and heart rate as well as an increase in weight relative to sham-irradiated animals. Female pups had significantly increased blood pressure with 300 mGy prenatal irradiation, decreased heart rate at 1000 mGy and were growth restricted over the course of the study period at 50, 100 and 1000 mGy. Male pups were only growth restricted at the highest dose of 1000 mGy. Overall these results indicate the programming of male offspring due to the stress of the necessary transportation of pregnant mothers and programming due to prenatal irradiation at higher doses of 300 and 1000 mGy.

2.2 Introduction

Pregnancy is a known period of sensitivity for the unborn child. This is due to the possibility of long-term implications following birth if a stress is experienced *in-utero*. This phenomenon, described initially described as the "Barker Hypothesis" is commonly referred to as developmental programming (Barker, 1990). The original hypothesis was based on a number of epidemiological studies, which reported a correlation between low birth weight or restricted growth in offspring and an increased incidence of cardiovascular disease in adulthood. Developmental programming can be broadly defined as a phenomenon in which adverse conditions during embryonic and fetal development can predispose the developing fetus to changes in adult phenotype (Barker et al., 2002).

Cardiometabolic pathologies of the offspring including hypertension and type II diabetes are well demonstrated phenotypic outcomes of developmental programming (reviewed by Alexander, 2006). Programming is often caused by maternal stress during pregnancy or other conditions that result in increased exposure of the developing offspring to glucocorticoids (GCs) such as cortisol (Moisiadis & Matthews, 2014; Nyirenda & Seckl, 1998). This has been demonstrated experimentally with both endogenous GC exposure (for example protein restriction models to induce maternal stress in rodents; Langley-Evans, 1996) and exogenous GC exposure (for example through treatment with a chemical GC such as dexamethasone; Dodic et al., 2002). Fetal programming of hypertension has been demonstrated experimentally using various triggers including hypoxia exposure of the pregnant mother, preeclampsia, maternal obesity and exposure to compounds which limit oxygen supply to the fetus (Mulder et al.,

2002; Wei et al., 2003; Drake & Reynolds, 2010; Roberts & Lain, 2002; Li et al., 2012; Thompson & Al-Hasan, 2012). A proposed mechanism of developmental programming of hypertension at the cellular level is an increased level of oxidative stress and reactive oxygen species (ROS) formation within the intrauterine environment. This has been supported by the fact that programming can be mechanistically linked to an elevated state of oxidative stress, whether directly (such as hypoxia exposure) or indirectly (e.g. through the trigger of the GC stress axis caused by maternal stress; Seckl, 2001). Numerous experimental studies have supported oxidative stress as a candidate mechanism for fetal programming (Luo et al., 2006; Thompson & Al-Hasan, 2012; Franco et al., 2002).

Pregnant women and their fetuses can receive exposure to ionizing radiation from numerous sources, including medical exposures from diagnostic radiography or nuclear medicine, occupational exposures including nuclear energy workers, medical professionals and airline workers as well as environmental exposures (Fattibene et al., 1999). Regardless of the route of exposure, virtually all human exposures to ionizing radiation are low dose in nature (<0.1 Gy). The biological effects of low levels of radiation have been the source of ongoing scientific debate for more than a century (Brenner et al., 2003). While there is a consensus that intermediate and high doses of ionizing radiation result in harmful biological effects, the effects at lower doses are not as clear. There are epidemiological data available regarding radiation effects as low as 0.1 Gy for acute exposures, with the effects below this level largely unclear (Brenner et al., 2003). Ionizing radiation produces oxidative stress, even at low doses, and therefore there is a potential role for ionizing radiation on developmental programming due to an

overlapping mechanism of action (Hall & Giaccia, 2012). There are reports of potential beneficial protective effects from low dose radiation exposure due to an adaptive level of oxidative stress (Miura, 2004; Feinendegen, 2005), however the role of low dose prenatal irradiation on developmental programming of cardiovascular disease has not been investigated.

Low dose ionizing radiation exposure during pregnancy in humans may also be of relevance for developmental programming due to its relationship with maternal stress, a well established trigger for programming. This is also common when considering prenatal irradiation studies in animal models, due to the presence of maternal stress, despite it often being unintentional. For example, restraint of the animal or treatment with an anesthetic is necessary to achieve precise, targeted irradiations. This is of concern, due to the known ability of physical restraint to induce a stress response in the animal (Stroth & Eiden, 2010). Irradiation facilities are often not located within the same facility of the animal housing unit, and therefore physical movement of the animal cages in order to irradiate them presents an additional possible source of maternal stress (Tuli et al., 1995). In order to minimize confounding levels of maternal stress during prenatal irradiations, attempts to habituate the animal are often accomplished through repetition of the stressful event (reviewed by Grissom & Bhatnagar, 2009).

We used a wildtype mouse model to study the effects of prenatal ionizing radiation exposure on offspring growth, blood pressure and heart rate. We predicted that offspring of irradiated mothers at high doses of irradiation (300 or 1000 mGy) would demonstrate a combination of growth restriction and hypertension due to programming. Due to the

specialized irradiation facility being located outside of the animal housing facility, the stress response of the required transportation was also studied. We predicted that acclimation of the animals to transport would minimize transportation stress and not impact developmental programming.

2.3 Materials and Methods

2.3.1 Animal Procedures

Male and female C57Bl/6J wildtype mice (Jackson Laboratories, Bar Harbor, Maine, USA), age 7-8 weeks, were given a one week period to acclimate to the animal holding room without disruption. Females were housed 5 per cage upon arrival at the facility until breeding. Breeder males were singly housed for the duration of the study. Animals were allowed food and water *ad-libitum* for the duration of the study and were maintained on a 12:12 hour light:dark cycle. All described animal procedures were approved by the Animal Research Ethics Board at McMaster University (AUP#15-11-26).

Female mice were moved to a male cage (2 females:1 male) and allowed to breed overnight. The following morning, females were removed from male cages and singly housed. The presence of vaginal plugs determined the first day of gestation. Prior to transport for irradiation, female mice were palpated by hand to confirm pregnancy. Only animals that were pregnant were irradiated as described below.

Following irradiations (see section 2.3.3), animals were returned to their housing room and remained singly housed. Mothers were allowed to pup without disruption (including cage changes) for a minimum of one week following birth of the litter. Pups were weaned

at 3-4 weeks of age, at which time sex was also determined. Up to three male or four female F1 pups were group housed in a cage. A maximum of two female and two male pups from a single mother were used for this study, to control for maternal effects.

A separate cohort of female mice was bred but remained in the animal facility for the duration of the pregnancy. The pups of these mothers represented a pure naïve control cohort ("No Transport") without any exposure to radiation (similar to sham), which allowed for comparison between sham-irradiated mice to test for the effect of the repeated transport acclimation protocol (see section 2.3.2 for description of the repeated transport protocol).

2.3.2 Acclimation to Transport

To acclimate the animal to the movement to the irradiation facility and minimize stress at the time of irradiation, animals were repeatedly transported to the irradiation facility prior to irradiations. Mice were transported 2-3 times per week starting one week prior to breeding and between gestational days 8 and 15. Cages were transported in a temperature-controlled vehicle to the irradiation facility and placed in the source room.

To monitor acute stress in the animals, blood serum levels of corticosterone (Cort) were measured. Blood was collected via cardiac puncture and Cort levels in blood serum were measured using a commercial ELISA kit (R&D Systems, Minneapolis, Minnesota, USA). This was done at various stages of the acclimation procedure, either with the pre-irradiation alarm (Figure 2.2) or with the pre-irradiation alarm muted (Figure 2.3). A negative control cohort "Naïve" included female mice that were never transported and

remained in the animal facility throughout the study. Cort levels were also measured at various steps of the transportation protocol, after acclimation to transport including immediately following arrival at the Taylor Source facility ("Driven"), after allowing mice to acclimate to the source room for 20 minutes ("Acclimated to Room"; both with and without the pre-irradiation alarm) and sham irradiated by opening and immediately closing the source ("Sham").

2.3.3 Irradiations

Pregnant females were irradiated at nominal doses of 5, 10, 50, 100, 300 and 1000 mGy ^{137}Cs gamma radiation (662 keV energy) using the Taylor Radiobiology Source at McMaster University at day 15 of gestation (Figure 2.1). Mice were transported in a temperature controlled vehicle to the irradiation facility in another building. Following transportation to the Taylor Source irradiation facility, mice were placed underneath the source for 20 minutes prior to irradiation in their home cage (without the pre-irradiation alarm, see section 2.3.2). Sham-irradiated animals were placed under the shielded source for 20 minutes and were then moved to the control room for the duration of the irradiation. Animals were restricted from food and water consumption for the period of irradiation (including sham-irradiated animals).

All doses were delivered at a nominal dose rate of 10 mGy min^{-1} with the fetal dose rate estimated to be 8.9 mGy min^{-1} (Table 2.1). Doses were verified using thermoluminescent dosimeters (TLDs; Mirion Technologies, Irvine, California, USA). TLDs were placed on bedding in an empty animal cage with the lid on, to estimate

attenuation through the lid. A total of 6 TLDs were placed at various locations in the beam window to account for lateral variation. To estimate fetal absorbed dose and radiation attenuation through the body of the pregnant mother, TLDs were implanted *in-situ* in pregnant female mice at day 15 of gestation. These mice were first euthanized, and two TLDs were surgically implanted in two locations: one subcutaneously and one in the approximate area of the fetus. Mice with implanted TLDs (n=3 mice) were placed under the source and irradiated in animal cages to simulate irradiations. Following irradiation, the TLDs were removed and the average dose rate (in mGy min^{-1}) measured. From the dose rate, the absorbed dose for each nominal dose was calculated based on the exposure time (Table 2.1).

2.3.4 Blood Pressure, Heart Rate and Weight Measurements

Blood pressure (BP) was non-invasively measured in F1 pups via tail-cuff plethysmography using the CODA8 High Throughput Non-invasive Blood Pressure system (Kent Scientific Corporation, Torrington, Connecticut, USA). Mice were restrained in Plexiglas tubes at an ambient temperature of ~27^{o}C. A tail-cuff with an attached photosensitive cell was placed at the base of the tail and changes in systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) of the animal were measured by the CODA software. Heart rate (HR) was calculated from the physiological traces that were collected during the described blood pressure measurements. This method of measuring blood pressure has been previously used in developmental studies and is a non-invasive method that poses minimal discomfort or stress to the animal (Feng et al., 2008; Longo et al., 2016). BP and HR measurements

were collected two to three times per week for each mouse from 5 -16 weeks of age. Animal weight was collected on a weekly basis following weaning until 16 weeks of age and animals were euthanized at 17 weeks of age.

2.3.5 Statistical Analysis

Throughout the text, data is presented as mean ± standard error of the mean (SEM) and $\alpha = 0.05$, unless otherwise stated. Statistical analysis was completed using IBM SPSS v.20 statistical software (IBM Corporation, Armonk, New York, USA). Comparison of transport acclimation groups via analysis of serum Cort levels was completed using one-way ANOVA.

For the three BP (systolic, diastolic and mean arterial pressure) and HR measures collected by the CODA system, 25 repeated cycles of measurements were collected each day per mouse and those that CODA determined were valid were used to calculate a median value. The CODA monitoring system automatically determined values to be true if they met a number of requirements including: a sufficient tail volume detected, appropriate time between the DBP and SBP measurements, a difference of at least 5 mm Hg between the SBP and DBP measurements and that the SBP is greater than the DBP.

Within each week of measurements, an average of the median values was calculated to generate a single value per week for each mouse. Outliers were determined to be any BP/HR value that was ± 2 SDs from the mean, and systematically removed from the dataset. An Analysis of Co-Variance (ANCOVA) was completed to compare differences between the radiation groups. The age of the mouse (in weeks) was used as a co-variate

for analysis and the between-subjects variable of radiation dose used as the fixed effect for ANCOVA analysis. Main effects were compared between radiation doses following Bonferroni adjustment for multiple comparisons. Weekly animal weights were compared between radiation doses using ANCOVA testing. For the three BP measures, HR and weight data, analyses were completed for male and female pups independently. Comparisons of the No Transport control group were only made with sham-irradiated pups.

2.4 Results

2.4.1 Dosimetry

Based on the TLDs, attenuation through the lid of the animal cage resulted in an actual dose rate of 9.1 mGy min^{-1} (approximately 10% attenuation) inside the cage. Implantation of TLDs into mice determined that there was a very minimal attenuation through the soft tissue of the pregnant mother, with a resulting dose rate of 8.9 mGy min^{-1} for both the skin surface and fetal dose. The calculated irradiation dosimetry for each nominal dose are provided in Table 2.1.,

2.4.2 Serum Corticosterone Levels

Serum Cort levels were measured in naïve mice that remained in the animal holding room and did not undergo any transportation. Serum Cort levels were also measured in mice after repeated transport acclimation including mice transported to the irradiation facility but without exposure to the pre-irradiation alarm and mice that were transported and exposed to the pre-irradiation alarm (Figure 2.2). There was a significant increase in

Cort concentrations in the cohort exposed to the pre-irradiation alarm, which had been manually muffled (one-way ANOVA, p=0.016).

Following muting of the pre-irradiation alarm, there were no significant differences in Cort levels between cohorts that remained in the animal holding room, animals driven to the irradiation facility, animals driven to the facility and allowed to acclimate to the room for 20 minutes, or animals that were allowed to acclimate and then sham irradiated with the pre-irradiation alarm turned off (one-way ANOVA, p=0.126; Figure 2.3). All irradiations of pregnant mothers involved animals that had transport acclimation, acclimation to the room for 20 minutes, and irradiation with the pre-irradiation alarm turned off.

2.4.3 Irradiation Effects on Blood Pressure, Heart Rate & Weight

In male pups, there was a significant decrease in all three blood pressure measures (SBP, DBP and MAP) at 10 mGy relative to male sham pups (Figure 2.4 panels A-C; ANCOVA), but not at other radiation doses. There was a significant decrease in HR at all doses except 50 mGy relative to sham pups ($p<0.0001$ for 10, 100, 300 and 1000 mGy) with the greatest reduction in HR at 1000 mGy (Figure 2.4 panel D). There was a significant increase in body weight in male pups at 10 mGy ($p<0.0001$) and 50 mGy ($p=0.004$) relative to male sham-irradiated pups (Figure 2.5 panel A; ANCOVA). A significant decrease in the weight of male pups was only detected at the highest tested dose of 1000 mGy ($p<0.0001$).

In female pups, there was a significant increase in all three blood pressure measures at 300 mGy, but not any other dose, compared to sham-irradiated female pups (Figure 2.6 panels A-C). There was a significant decrease in HR in female pups at the highest dose of 1000 mGy relative to 10 mGy ($p=0.003$), 100 mGy ($p<0.0001$) and 300 mGy ($p<0.0001$) but not with sham irradiation (ANCOVA; Figure 2.6 panel D). A significant decrease in body weight in female pups was detected at 50 mGy ($p<0.0001$), 100 mGy ($p<0.0001$) and 1000 mGy ($p<0.0001$) compared to female sham-irradiated pups (Figure 2.5 panel B; ANCOVA).

2.4.4 Effects of Transport

When comparing sham-irradiated male pups to the male No Transport pups, significant differences were detected for all measured variables (refer to Table S.2.1 for a summary of transport effects). Transportation of the mother resulted in an increase in all three blood pressure variables ($p<0.001$, $p=0.004$ and $p=0.02$ for SBP, DBP and MAP respectively; Figure 2.4 panels A-C) and HR ($p=0.018$ ANCOVA; Figure 2.4 panel D). There was also a significant decrease in weight in male sham mice from transported mothers relative to male No Transport pups ($p=0.003$; Figure 2.5 panel A).

In female pups, the only measured variables significantly affected by the acclimation protocol (i.e. statistical difference between sham and No Transport controls) were DBP which was significantly increased in the female sham pups ($p=0.007$; Figure 2.6 panel B) and weight, which was decreased in sham-irradiated female pups ($p=0.048$; Figure 2.5s panel B).

2.5 Discussion

The aim of this study was to investigate the effects of prenatal exposure to ionizing radiation in mice on postnatal measures of growth, blood pressure and heart rate in offspring. Because the radiation source was located in specialized facilities, outside of central animal facilities, we had to transport animals. Thus, we included an assessment of the corticosterone levels of animals in our acclimation protocols and No Transport controls to determine effects of transport. Despite attempts to minimize stress, and an acclimation protocol that did not significantly elevate serum corticosterone levels, comparisons of BP, HR, and weight in pups from sham-irradiated controls and No Transport controls suggests unintended effects due to transportation of mothers. The strongest evidence for cardiometabolic programming from irradiation occurred at high doses, particularly at 1000 mGy, where a significant decrease in HR and growth was found in both genders. Female pups also had increased BP measures (SBP, DBP and MAP) at 300 mGy.

Transportation of mothers was an unavoidable component of the study design due to the irradiation facility being in a different building. Fetal programming of hypertension has been well documented to be triggered by maternal stress and subsequent release of GCs and therefore limiting stress of the pregnant mothers would avoid confounding the effects of prenatal irradiation (reviewed by Seckl, 2001). Although care was taken to minimize abrupt movement of animal cages during transportation, it was understood that these transportation procedures were likely stressful due to movement of the animal

cages, changes in temperature, noises, and/or smells/environment. For example, tilting animal cages 45^{o} has been used as a means of inducing chronic unpredictable stress in rodent studies, illustrating the risk of stress that is present during transportation of animal cages (Ducottet & Belzung, 2005). The potential programming effects of the transportation in this study were surprising when considering the Cort analysis. Following muting of the pre-irradiation alarm, there was no significant increase in stress in the animals based on the serum Cort levels at the time of sacrifice, which were not different from naïve animals. Perhaps the repetitive transportation of animals 2-3 times per week may have resulted in a more mild, chronic duration of stress. Chronic stress during the prenatal period has been implicated in the programming of offspring. For example, Maccari et al. (2003) studied the effects of prenatal restraint stress by placing Sprague Dawley rats in conical cylinders from gestational day 11 until delivery. The authors reported gender-specific effects of this prenatal restraint stress paradigm including an increased responsiveness of the hypothalamic-pituitary-adrenal axis dysfunctions as well as dysfunctions in the metabolic/immune system and offspring behaviour (reviewed by Darnaudéry & Maccari, 2008).

The No Transport cohort served as a pure naïve control cohort, which allowed for observation of the effects of transportation by comparing to sham-irradiated pups of the respective gender. There was evidence for programming in offspring from transportation alone, based on a significant decrease in body weight in sham pups of both genders relative to No Transport controls. Low birth weight and growth restriction have been well characterized as a phenotype of fetal programming (reviewed by Harris & Seckl, 2011).

Compared to No Transport controls, there was also an increase in all three BP measures and HR in male pups from the sham group, as well as increased DBP in female pups from the sham group. Hypertensive offspring due to programming has also been demonstrated in numerous studies (for example Samuelsson et al., 2008), further supporting that the transported mice were programmed.

Interestingly, programming appeared to vary based on the gender of the offspring. Animal measurements were analyzed independently by gender due to the differences in baselines values for each measure between genders. There is growing evidence that fetal programming can differentially affect genders. Gender differences in the fetal programming of hypertension have been reported in both human clinical data as well as animal studies (reviewed by Grigore et al., 2008). One proposed explanation for the gender disparity has been the role of sex hormones on programming. Ojeda et al. (2006) reported elevated testosterone levels in hypertensive adult rats that were experimentally programmed. The authors were able to reverse these programming effects by castration at 10 weeks of age, which would support that testosterone plays a role in the hypertensive phenotype of growth-restricted male offspring. This is hypothesized to be a modulatory role of testosterone on the renin-angiotensin system, a key regulator of both blood pressure and blood volume within the body.

A single 10 mGy irradiation at gestational day 15 appeared to have attenuated the programming effects caused by transportation in sham-irradiated male pups. For all endpoints measured, the 10 mGy irradiation group was returned to the values in the male No Transport control mice. This was the first study to investigate low dose irradiation

effects *in-utero* on postnatal cardiometabolic measures and these data suggest protective effects with 10 mGy irradiation in male pups. Although the mice in this study were irradiated after the completion of organogenesis, there have been other studies that have demonstrated protective effects of low dose irradiations during pregnancy. For example, Wang et al. (1998) reported a significant increase in the rate of living fetuses and decreased congenital malformation incidence when mice challenged with a 5 Gy irradiation on gestational day 12 were pre-treated with a priming dose of 300 mGy 24 priors to the challenge dose. The priming dose of 300 mGy in this study was clearly adaptive, as the decreased congenital malformation incidence was relative to mice that only received the 5 Gy challenge dose. Similarly, a 300 mGy priming irradiation on gestational day 10 was protective from the teratogenic effects of a subsequent challenge irradiation of 4 Gy 24 hours later in mice with varying Trp53 tumor suppressor protein status (Mitchel et al., 2002). Despite the difference in study design, particularly the presence of a very high challenge dose in the previous studies, it is important to realize that low dose irradiations often do not often produce observable effects alone. Therefore, the ability of a low dose radiation treatment to attenuate an additional stressor is an observable adaptive response due to low dose irradiation. Further research is required to identify the mechanism underlying this low dose radiation adaptive response.

The observed adaptive response with 10 mGy irradiation and transportation stress in male pups provide interesting considerations for future research. Although attempts were made to minimize stress caused by transportation of animal cages, it is evident that transportation stress must be carefully considered in study design. The repetitive nature of

the transportation protocol which was in an attempt to acclimate the animal, modification in the transportation protocol may be an area of modification for future studies.

2.6 Acknowledgements

The authors thank Devon Jones and Abigail Lee for assistance with blood pressure measurement collection and Dr. Sujeenthar Tharmalingam for assistance with blood serum Cort analysis. The authors also thank Robin Howe and staff at the Central Animal Facility at McMaster University for their tireless support with animal care. Irina Toore and the staff at Kent Scientific provided technical support with the CODA8 system. Funding was provided by Bruce Power to T.C. Tai and Joanna Y. Wilson, as well as a Collaborative Research and Development Grant from the Natural Sciences and Engineering Council of Canada (NSERC) to T.C. Tai, Joanna Y. Wilson and Douglas R. Boreham. Shayenthiran Sreetharan and Adomas Kulesza were supported by NSERC's Canada Graduate Scholarship - Master's Program scholarship.

2.7 References

Alexander, B.T., 2006. Fetal programming of hypertension. AJP: Regulatory, Integrative and Comparative Physiology 290, R1–R10.

Barker, D., Eriksson, J., Forsén, T., Osmond, C., 2002. Fetal origins of adult disease: strength of effects and biological basis. International Journal of Epidemiology 31, 1235–1239.

Barker, D.J., 1990. The fetal and infant origins of adult disease. BMJ 301, 1111–1111.

Brenner, D.J., Doll, R., Goodhead, D.T., Hall, E.J., Land, C.E., Little, J.B., Lubin, J.H., Preston, D.L., Preston, R.J., Puskin, J.S., Ron, E., Sachs, R.K., Samet, J.M., Setlow, R.B., Zaider, M., 2003. Cancer risks attributable to low doses of ionizing radiation: Assessing what we really know. Proceedings of the National Academy of Sciences 100, 13761–13766.

Darnaudéry, M., Maccari, S., 2008. Epigenetic programming of the stress response in male and female rats by prenatal restraint stress. Brain Research Reviews 57, 571–585.

Dodic, M., Abouantoun, T., O'Connor, A., Wintour, E.M., Moritz, K.M., 2002. Programming Effects of Short Prenatal Exposure to Dexamethasone in Sheep. Hypertension 40, 729–734.

Drake, A.J., Reynolds, R.M., 2010. Impact of maternal obesity on offspring obesity and cardiometabolic disease risk. Reproduction 140, 387–398.

Ducottet, C., Belzung, C., 2005. Correlations between behaviours in the elevated plus-maze and sensitivity to unpredictable subchronic mild stress: evidence from inbred strains of mice. Behavioural Brain Research 156, 153–162.

Fattibene, P., Mazzei, F., Nuccetelli, C., Risica, S., 1999. Prenatal exposure to ionizing radiation: sources, effects and regulatory aspects. Acta Paediatrica 88, 693–702.

Feinendegen, L.E., 2005. Evidence for beneficial low level radiation effects and radiation hormesis. The British Journal of Radiology 78, 3–7.

Feng, M., Whitesall, S., Zhang, Y., Beibel, M., Alecy, L.D., DiPetrillo, K., 2008. Validation of Volume-Pressure Recording Tail-Cuff Blood Pressure Measurements. American Journal of Hypertension 21, 1288–1291.

Franco, M. do C.P., Dantas, A.P.V., Akamine, E.H., Kawamoto, E.M., Fortes, Z.B., Scavone, C., Tostes, R.C.A., Carvalho, M.H.C., Nigro, D., 2002. Enhanced oxidative stress as a potential mechanism underlying the programming of hypertension in utero. J. Cardiovasc. Pharmacol. 40, 501–509.

Grigore, D., Ojeda, N.B., Alexander, B.T., 2008. Sex differences in the fetal programming of hypertension. Gender Medicine 5, S121–S132.

Grissom, N., Bhatnagar, S., 2009. Habituation to repeated stress: Get used to it. Neurobiology of Learning and Memory 92, 215–224.

Hall, E.J., Giaccia, A.J., 2012. Radiobiology for the radiologist. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia

.

Harris, A., Seckl, J., 2011. Glucocorticoids, prenatal stress and the programming of disease. Hormones and Behavior 59, 279–289.

Langley-Evans, S.C., 1996. Intrauterine programming of hypertension in the rat: Nutrient interactions. Comparative Biochemistry and Physiology Part A: Physiology 114, 327–333.

Li, Y., Gonzalez, P., Zhang, L., 2012. Fetal stress and programming of hypoxic/ischemic-sensitive phenotype in the neonatal brain: Mechanisms and possible interventions. Progress in Neurobiology 98, 145–165.

Longo, M., Refuerzo, J.S., Mann, L., Leon, M., Moussa, H.N., Sibai, B.M., Blackwell, S.C., 2016. Adverse Effect of High-Fat Diet on Metabolic Programming in Offspring Born to a Murine Model of Maternal Hypertension. American Journal of Hypertension hpw088.

Luo, Z.C., Fraser, W.D., Julien, P., Deal, C.L., Audibert, F., Smith, G.N., Xiong, X., Walker, M., 2006. Tracing the origins of “fetal origins” of adult diseases: Programming by oxidative stress? Medical Hypotheses 66, 38–44.

Maccari, S., Darnaudery, M., Morley-Fletcher, S., Zuena, A.R., Cinque, C., Van Reeth, O., 2003. Prenatal stress and long-term consequences: implications of glucocorticoid hormones. Neuroscience & Biobehavioral Reviews 27, 119–127.

Mitchel, R.E.J., Dolling, J.-A., Misonoh, J., Boreham, D.R., 2002. Influence of Prior Exposure to Low-Dose Adapting Radiation on Radiation-Induced Teratogenic Effects in Fetal Mice with Varying *Trp53* Function. Radiation Research 158, 458–463.

Miura, Y., 2004. Oxidative Stress, Radiation-Adaptive Responses, and Aging. Journal of Radiation Research 45, 357–372.

Moisiadis, V.G., Matthews, S.G., 2014. Glucocorticoids and fetal programming part 1: outcomes. Nature Reviews Endocrinology 10, 391–402.

Mulder, E.J.., Robles de Medina, P.., Huizink, A.., Van den Bergh, B.R.., Buitelaar, J.., Visser, G.H.., 2002. Prenatal maternal stress: effects on pregnancy and the (unborn) child. Early Human Development 70, 3–14.

Nyirenda, M.J., Seckl, J.R., 1998. Intrauterine events and the programming of adulthood disease: the role of fetal glucocorticoid exposure (Review). International Journal of Molecular Medicine.

Ojeda, N.B., Grigore, D., Yanes, L.L., Iliescu, R., Robertson, E.B., Zhang, H., Alexander, B.T., 2006. Testosterone contributes to marked elevations in mean arterial pressure in adult male intrauterine growth restricted offspring. AJP: Regulatory, Integrative and Comparative Physiology 292, R758–R763.

Roberts, J.M., Lain, K.Y., 2002. Recent Insights into the Pathogenesis of Pre-eclampsia. Placenta 23, 359–372.

Samuelsson, A.-M., Matthews, P.A., Argenton, M., Christie, M.R., McConnell, J.M., Jansen, E.H.J.M., Piersma, A.H., Ozanne, S.E., Twinn, D.F., Remacle, C., Rowlerson, A., Poston, L., Taylor, P.D., 2008. Diet-Induced Obesity in Female Mice Leads to Offspring Hyperphagia, Adiposity, Hypertension, and Insulin Resistance: A Novel Murine Model of Developmental Programming. Hypertension 51, 383–392.

Seckl, J.R., 2001. Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. Molecular and Cellular Endocrinology 185, 61–71.

Stroth, N., Eiden, L.E., 2010. Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on pituitary adenylate cyclase-activating polypeptide signaling. Neuroscience 165, 1025–1030.

Thompson, L.P., Al-Hasan, Y., 2012. Impact of Oxidative Stress in Fetal Programming. Journal of Pregnancy 2012, 1–8.

Tuli, J.S., Smith, J.A., Morton, D.B., 1995. Stress measurements in mice after transportation. Laboratory Animals 29, 132–138.

Wang, B., Ohyama, H., Nose, T., Itsukaichi, H., Nakajima, T., Yukawa, O., Odaka, T., Tanaka, K., Kojima, E., Yamada, T., Hayata, I., 1998. Adaptive Response in Embryogenesis: I. Dose and Timing of Radiation for Reduction of Prenatal Death and Congenital Malformation during the Late Period of Organogenesis. Radiation Research 150, 120.

Wei, J.-N., Sung, F.-C., Li, C.-Y., Chang, C.-H., Lin, R.-S., Lin, C.-C., Chiang, C.-C., Chuang, L.-M., 2003. Low Birth Weight and High Birth Weight Infants Are Both at an Increased Risk to Have Type 2 Diabetes Among Schoolchildren in Taiwan. Diabetes Care 26, 343–348.

Nominal Dose (mGy)	Dose in Cage (mGy)	Skin Surface Dose (mGy)	Fetal Dose (mGy)
0	0	0	0
5	4.6	4.4	4.4
10	9.1	8.9	8.9
50	45.6	44.5	44.3
100	91.2	88.9	88.5
300	273.7	266.8	265.6
1000	912.3	889.3	885.2

Table 2.1: Calculated irradiation dosimetry. The dose in the animal cage was measured by placing thermoluminescent dosimeters (TLDs) in an empty animal cage with the lid on to calculate attenuation through the lid. TLDs were also surgically implanted in pregnant mice euthanized at gestational day 15. One TLD was implanted subcutaneously under the skin to estimate skin surface dose and one in the approximate location of the fetus (n=3 mice). Following measurement of the dose rate (in mGy min^{-1}) using TLDs, the dose rate was multiplied by exposure time to calculate the doses reported in the table. Multiple cages were placed under the beam window to account for differences in dose rate due to lateral variation.

		Estimated Marginal Mean		p-value
		Sham	No Transport	
Male				
	N	9	5	
	Systolic Pressure (mm Hg)	101.79 (0.80)	96.76 (1.07)	<0.001*
	Diastolic Pressure (mm Hg)	74.51 (0.75)	70.80 (1.00)	0.004*
	Mean Arterial Pressure (mm Hg)	83.08 (0.75)	79.26 (0.99)	0.02*
	Heart Rate (bpm)	367.48 (8.50)	333.91 (11.19)	0.018*
	Weight (g)	23.67 (0.16)	24.54 (0.24)	0.003*
Female				
	N	8	6	
	Systolic Pressure (mm Hg)	99.95 (0.91)	99.30 (1.06)	0.64
	Diastolic Pressure (mm Hg)	74.51 (0.83)	71.03 (0.96)	0.007*
	Mean Arterial Pressure (mm Hg)	82.26 (0.83)	80.18 (0.95)	0.10
	Heart Rate (bpm)	301.16 (6.83)	317.87 (7.90)	0.11
	Weight (g)	19.05 (0.89)	19.43 (0.17)	0.048*

Table S2.1: Summary of the effects of transport protocol. Sham irradiated pregnant females were repeatedly transported to the irradiation facility as a means of acclimation to the stress of transport, left for 20 minutes to acclimate to the room, and irradiated without pre-irradiation alarms. Measures of blood pressure, heart rate and weight from sham-irradiated pups were compared to pure-naïve pups generated from mothers that were never transported out of the animal facility (No Transport). Comparisons were made following ANCOVA analysis with estimated marginal means reported; values indicate mean (SEM). Significant pairwise difference between Sham and No Transport for the respective measure ($p<0.05$) indicated *.

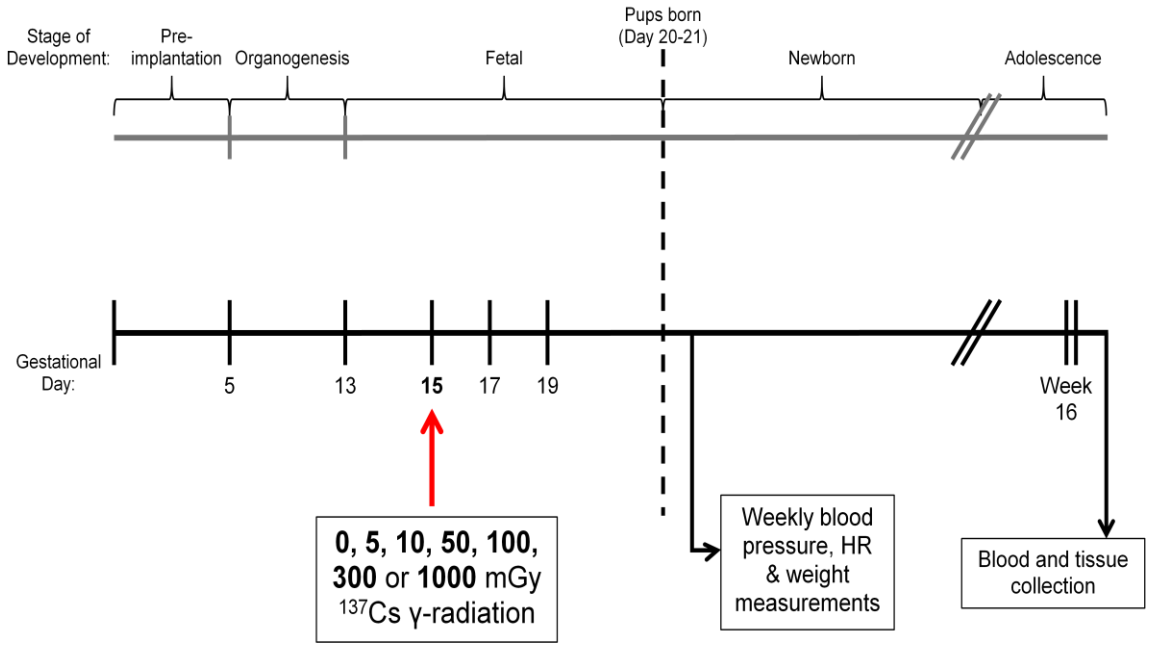

Figure 2.1: Experimental design overview. Gestational days indicated with day 1 being the day female mice were removed from males. Females were irradiated on gestational day 15 in this study. Blood pressure, heart rate and weight measurements began following weaning of pups at roughly 4-5 weeks of age. Animals were euthanized following a last set of measurements completed at 16 weeks of age. This animal study was approved by the McMaster Animal Research Ethics Board (AUP#15-11-26). HR = heart rate.

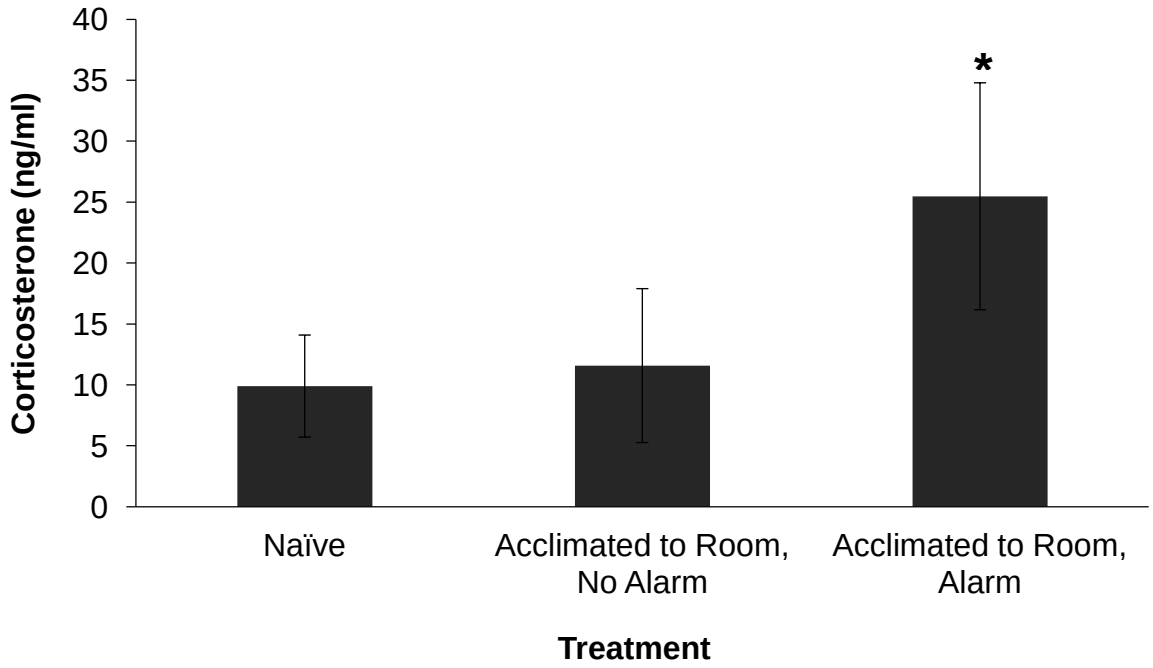

Figure 2.2: Effects of acclimation and irradiation alarm on corticosterone levels. Corticosterone concentrations (ng ml^{-1}) were measured in the serum of mice that were not acclimated to transport ("Naïve"; n=4), acclimated to transport but not exposed to the muffled pre-irradiation alarm ("Acclimated to Room, No Alarm"; n=4) or mice that were acclimated to transport and exposed to the muffled alarm ("Acclimated to Room, Alarm"; n=5). Error bars indicate standard deviation. * indicates statistical pairwise difference from the other treatments following one-way ANOVA testing (p=0.016).

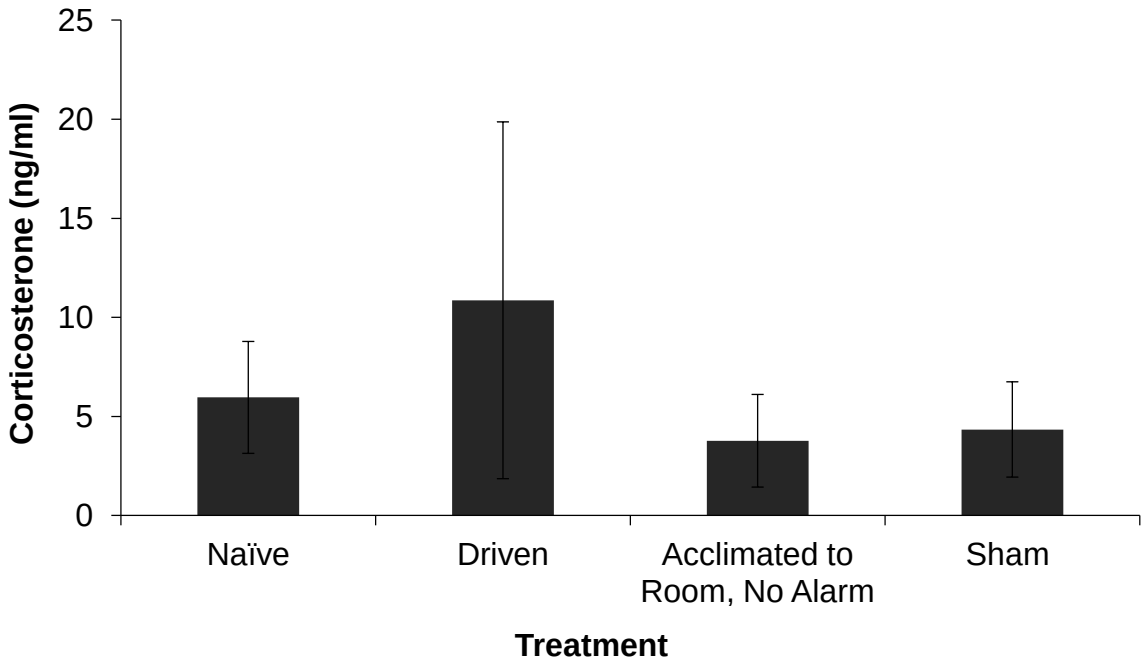

Figure 2.3: Effects of acclimation protocol on corticosterone levels. Corticosterone concentrations (ng ml^{-1}) were measured in the serum of mice that were not acclimated to transport ("Naïve"; n=3), driven to the facility and immediately sacrificed ("Driven"; n=5), driven and placed under the source for 20 minutes without irradiation ("Acclimated to Room, No Alarm"; n=6) or driven, acclimated for 20 minutes then sham irradiated by opening and immediately closing the source ("Sham"; n=6). Error bars indicate standard deviation. Statistical significance was not detected between groups following one-way ANOVA testing (p=0.126).

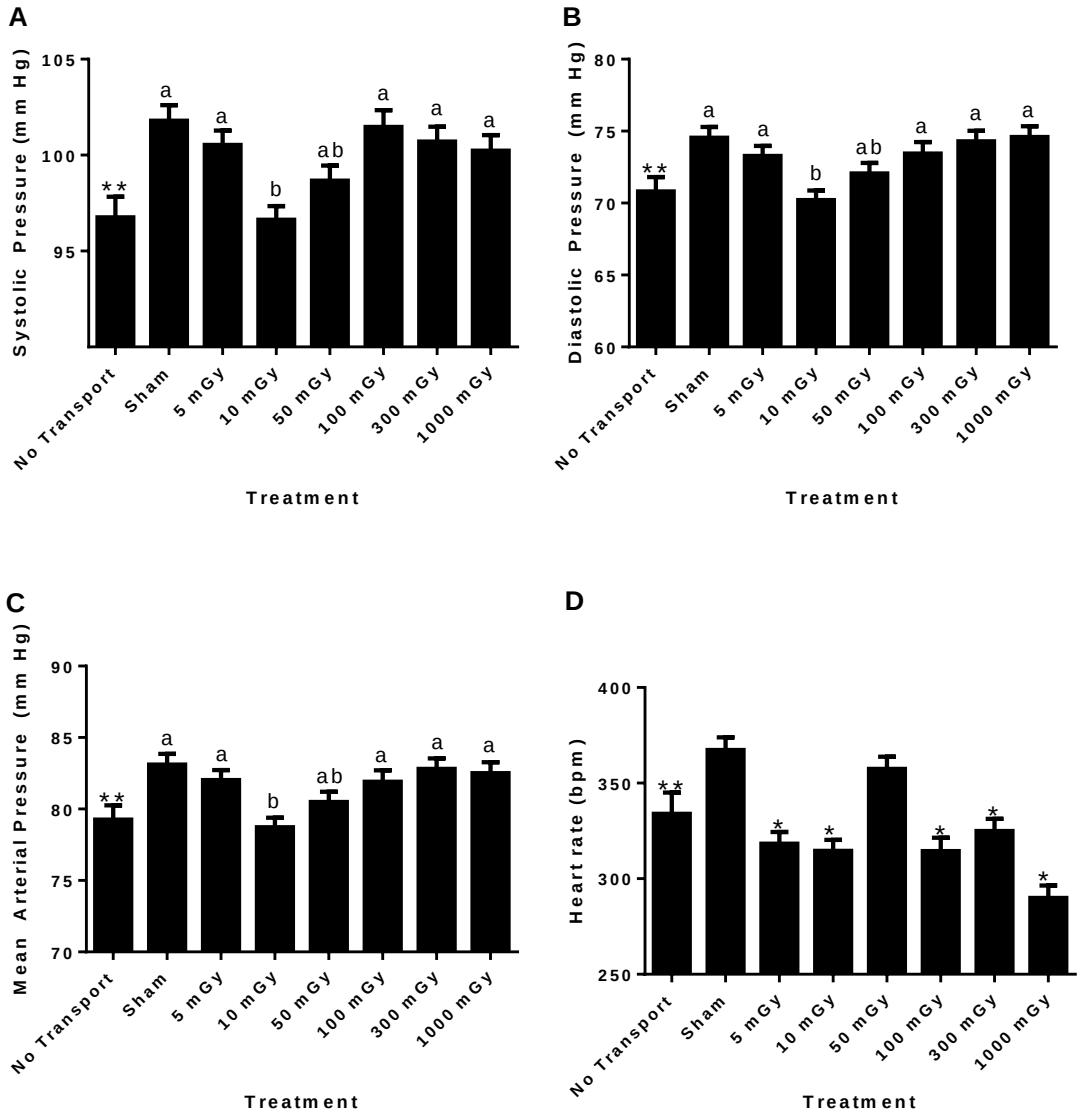

Figure 2.4: Blood pressure and heart rate in male pups. Blood pressure measurements (in mm Hg) of (A) systolic pressure, (B) diastolic pressure and (C) mean arterial pressure and (D) heart rate (in bpm) measurements of male mice irradiated prenatally at various radiation doses (mGy). Values are estimated marginal means following ANCOVA analysis, with error bars indicating SEM. Measurements were collected for consecutive weeks, from 5 to 16 weeks of age. Different letters above error bars indicate significant pairwise differences following significance detected for the between-subjects factor of radiation dose. For heart rate, * indicates significant pairwise difference from sham-irradiated pups. ** indicates significant pairwise difference between No Transport and sham. Sample sizes (number of mice) for each radiation cohort: sham = 9, 5 mGy = 11, 10 mGy = 11, 50 mGy = 10, 100 mGy = 11, 300 mGy = 10 and 1000 mGy = 9.

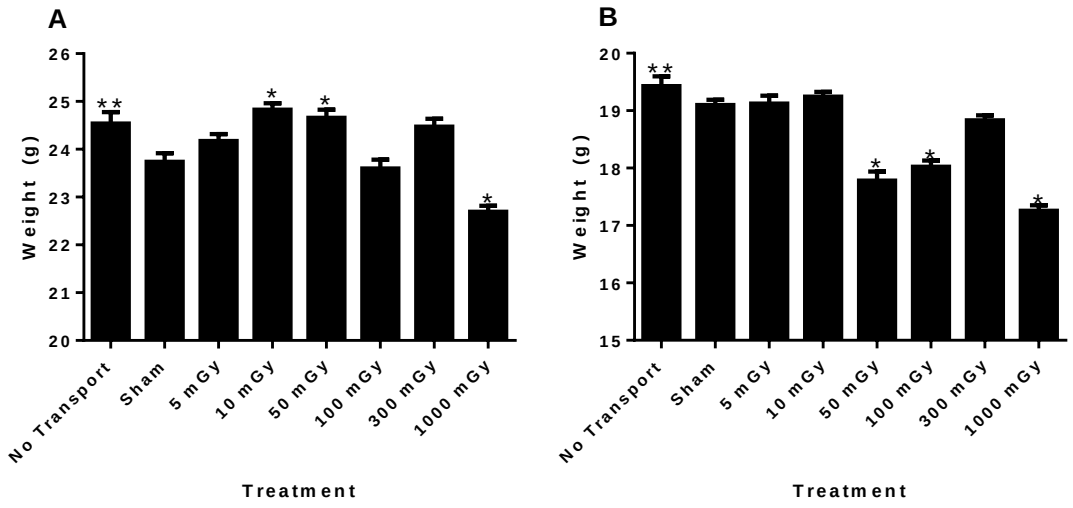

Figure 2.5: Animal weight in (A) male and (B) female F1 pups exposed to various levels of ionizing radiation during fetal development. Measurements were collected for consecutive weeks, from 4 to 16 weeks of age. Values are estimated marginal means following ANCOVA analysis, with error bars indicating SEM. * indicates significant pairwise difference compared to sham-irradiated pups. ** indicates significant pairwise difference of No Transport compared to sham-irradiated pups of the respective gender.

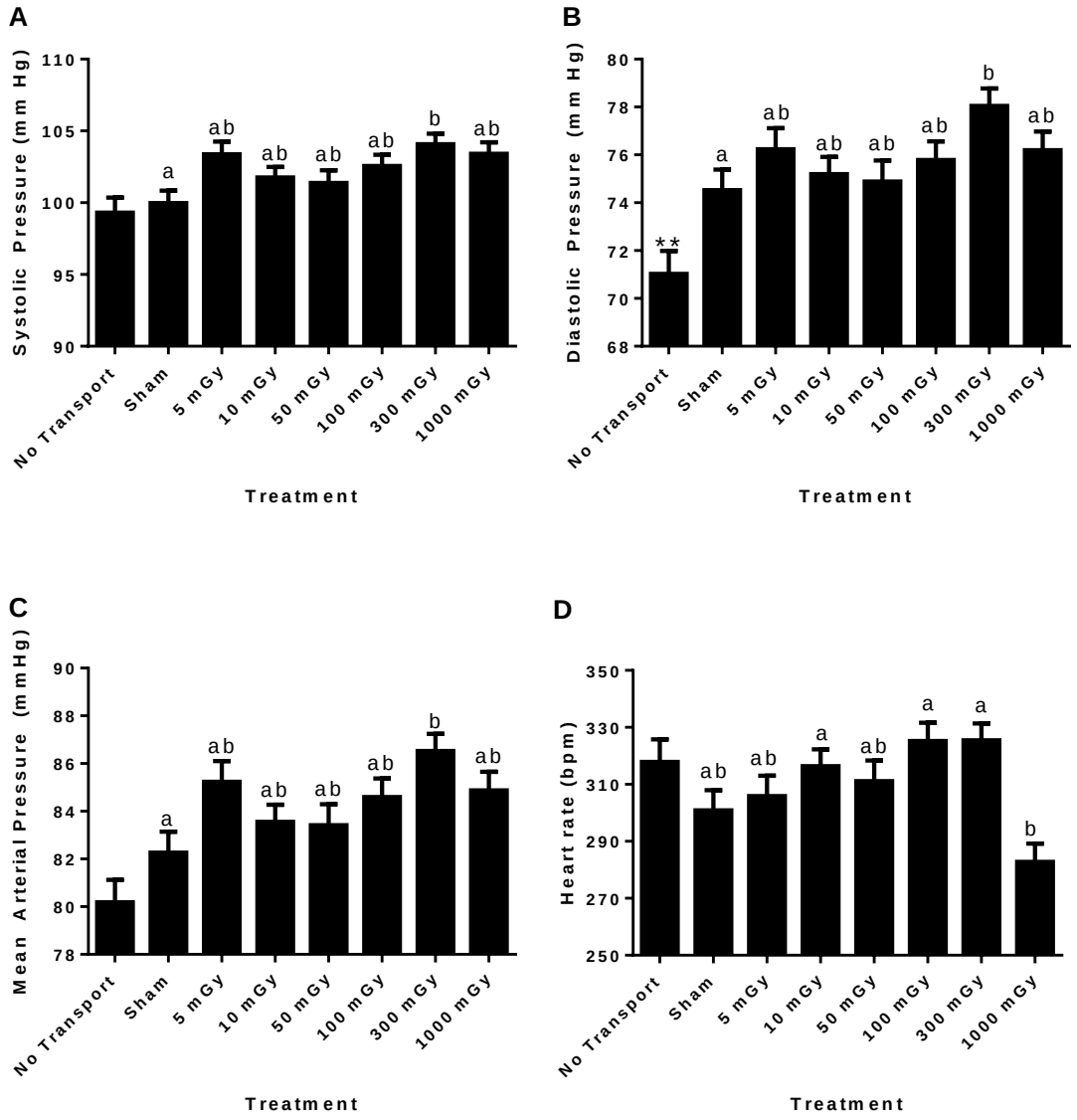

Figure 2.6: Blood pressure and heart rate in female pups. Blood pressure measurements (in mm Hg) of (A) systolic pressure, (B) diastolic pressure and (C) mean arterial pressure and (D) heart rate (in bpm) in female mice irradiated prenatally at various radiation doses (mGy). Values are estimated marginal means following ANCOVA analysis, with error bars indicating SEM. Measurements were collected for consecutive weeks, from 5 to 16 weeks of age. Different letters above error bars indicate significant pairwise differences following significance detected for the between-subjects factor of radiation dose. ** indicates significant pairwise difference between No Transport and sham. Sample sizes (number of mice) for each radiation cohort: sham = 8, 5 mGy = 8, 10 mGy = 12, 50 mGy = 7, 100 mGy = 10, 300 mGy = 12 and 1000 mGy = 10.

Chapter 3

Discussion and conclusions

3.1 Summary of Irradiation Effects

This thesis investigated the dose response relationship of ionizing radiation exposure during fetal development on post-natal growth and cardiovascular endpoints. We used a wildtype mouse strain of the commonly studied C57Bl mouse model and irradiated on gestational day 15 with ^{137}Cs gamma radiation at doses ranging from 5 - 1000 mGy. Pup weight, blood pressure and heart rate were measured weekly following birth until 16 weeks of age. Studies that use other stressors (e.g. hypoxia exposure to the pregnant mother, pre-eclampsia, protein restriction during pregnancy) during pregnancy have demonstrated a phenotype of growth restriction and hypertension in offspring that have undergone fetal programming (Li et al., 2012; Roberts & Lain, 2002; Wei et al., 2003; Langley-Evans, 1996). The role of prenatal ionizing radiation in producing a similar phenotype had not been previously investigated.

Evidence of growth restriction over the study period was detected at 50, 100 and 1000 mGy in female pups. Growth restriction in male pups was only observed at 1000 mGy. There was a significant increase in all three BP measures (SBP, DBP and MAP) with 300 mGy irradiation in female pups. No increase in blood pressure was detected with any dose in male pups. There was a significant decrease in HR in both genders irradiated at 1000 mGy. The impacts of prenatal irradiation on offspring growth and cardiovascular endpoints in this study, particularly at higher doses of ionizing radiation (>0.10 Gy) provide preliminary evidence of fetal programming due to ionizing radiation exposure during pregnancy.

3.2 Growth Restriction and Persistence of Response

One reported offspring phenotype of developmental programming includes low birth weight and possible permanent reduction in body weight (Godfrey & Barker, 2001). This reduction in growth (either at birth or over the course of postnatal development) has been reported in other programming studies (reviewed by Holemans et al., 2003), with one possible explanation involving regulatory factors influencing abnormal remodelling of blood supply to the fetus and possible regulation by local oxygen partial pressure (Maulik et al., 2006). Intrauterine growth restriction has also been reported in prenatal irradiation studies. For example, Murphree & Pace (1960) reported growth reduction at all tested doses (1.10 - 2.20 Gy of ^{60}Co gamma radiation) in rats irradiated at various points in gestation. A well defined mechanism for growth restriction in offspring due to prenatal exposure to ionizing radiation has yet to be conclusively recognized.

Although both low birth weight and decreased growth over the course of postnatal development have been reported in programming studies, a decreased birth weight is not necessarily always coupled with reduced growth. This is possible when a programmed offspring experiencing intrauterine growth restriction is born with a low birth weight, but subsequently "catches" up in size by accelerating growth, with this adequately named the "catch-up" hypothesis (Hales & Barker, 1992; Cianfarani et al., 1999). Fetal programming from a broader evolutionary perspective involves developmental plasticity of the fetus adapting to a stressful intrauterine environment and subsequent reduction in weight at birth (Kapoor et al., 2006). The catch-up hypothesis in general is based on the idea that if

the programmed offspring is no longer exposed to a maladaptive environment, catch-up growth occurs to compensate for the low birth weight following birth into a non-harmful environment (Hales et al., 1997).

The catch-up phenotype hypothesis and idea of catch-up growth are important to consider when discussing weight data. There are reports of persistent growth restriction over the course of the study period in previous studies that prenatally irradiated animals during the fetal stage of development from 14-20 days of gestation in mice (Streffer & Molls, 1987; UNSCEAR, 1977). Jensh et al. (1995) reported persistent growth restriction in offspring, up to 18 weeks of age (late adolescence), from Wistar rats irradiated prenatally with 2.0 Gy x-ray irradiation. Similarly, Murphree & Pace (1960) reported lower average weight in Sprague Dawley rats at all tested doses of 1.10, 1.50 and 2.20 Gy of ^{60}Co gamma radiation until the end of the ~13 week study period (adolescence). Irradiation of mice with 2.81 Gy of x-ray irradiation at 17 days of gestation also resulted in persistent growth reduction at the end of the study period (~11 weeks or adolescence; Nash & Gowen, 1962). These studies that reported persistent growth restriction in pups irradiated prenatally during fetal development all used higher doses of radiation (>0.1 Gy as described in Chapter 2). It is important to note that these studies did not follow the offspring into late adulthood.

We similarly observed persistent growth reduction at the highest tested nominal dose of 1.0 Gy in both genders and at 100 mGy in female pups. This thesis however, is the first to report persistent growth reduction at a lower dose of 50 mGy. With these observed growth reductions over the course of the study period in this thesis, it is assumed that

these mice were also born with a low birth weight, although this was not measured in this thesis. We chose to avoid the disruption of newborn mothers that would be required to measure birth weight in order to minimize cannibalism, a persistent issue in this study. We do not have evidence for catch-up growth based on the weight data, as mice remained growth restricted at the end of study period in this thesis (16 weeks).

When considering prenatal irradiation studies, there are a number of other offspring endpoints that have been investigated, such as offspring behaviour, in which the persistence of effects have been reported to be variable. Minamisawa and Hirokaga (1996) reported effects on open field activityin offspring at doses as low as 0.10 Gy of ^{137}Cs gamma radiation. This was observed in early and mid adulthood of offspring (6-7 months and 12-13 months respectively), but was not reported at late adulthood (19-20 months). Surprisingly, when the authors investigated higher doses of 0.50 and 1.0 Gy with the remainder of the study conditions the same, the effects were only observed in late adulthood (19-20 months; Minamisawa & Hirokaga, 1995). This would suggest that prenatal irradiations effects on the endpoint of offspring behaviour are not persistent at all doses, and perhaps only observable at late adulthood if the fetal absorbed dose is sufficiently high enough.

Although offspring behaviour was not an endpoint that was investigated in this thesis, this idea of a potential threshold dose for prenatal irradiation responses is interesting when considering the growth effects we reported. The results in this thesis suggest a lower threshold dose of 50 mGy in female pups, although intriguingly growth restriction was not observed at 300 mGy in female pups. Additionally, it is important to note that

these growth effects were consistent over the study period of the thesis, however the total duration of this study (16 weeks) is not as long as the studies by Minamisawa et al., who followed offspring for 20 months. We speculated that if effects in this thesis were persistent until 16 weeks of age (approximately adolescent stage of life in mice), that it would indicate a permanent phenotype in the offspring. The behavioural results of Minamisawa et al. would suggest that lengthening the post-natal study period to 20 months (late adulthood in mice), may be an interesting avenue for future research, although clearly entailing a much more labour-intensive study.

3.3 Blood Pressure and Heart Rate Effects

Blood pressure in the offspring of mice irradiated *in-utero* has not previously been investigated. Hypertension in offspring has been reported in other studies using established methods of programming (for example in rats that were under caloric or total protein restriction; Ozaki et al., 2001; Manning & Vehaskari, 2001), and therefore selected as a measure in this thesis. The only observed BP response due to irradiation was at 300 mGy in female pups, which resulted in a significant increase in SBP, DBP and MAP in female pups relative to sham irradiated pups. A hypertensive phenotype in offspring has been well characterized in other programming studies (reviewed by Alexander, 2006), although this was the first report with the use of prenatal irradiation as the stressor.

Similar to the reduction in weight observed in both genders at 1000 mGy, a significant decrease in HR was observed in both genders. Although there were decreases in HR at

other doses in male pups, the greatest decrease was observed at 1000 mGy. The consistent decrease in HR in both genders was notable because it was consistent with a significant decrease in animal weight in both genders at the same dose. This similarity of both weight restriction and decreased heart rate in both genders provides the strongest evidence for programming effects at the highest tested dose of 1000 mGy in this study.

Despite evidence for cardiovascular programming of BP and HR at high doses of radiation, possible clinical implications for a pathological phenotype are relatively weak. For instance, the increase in SBP for female pups from the sham (99.97 ± 0.88 mm Hg; mean ± SEM) to the 300 mGy (104.08 ± 0.73 mm Hg) treatment groups represents a ~4% increase in BP. The SBP values in this study are roughly comparable to human SBP values, with one proposed range for normal human resting SBP being between 90 and 120 mm Hg (Robinson & Brucer, 1939). If this approximate range for normal SBP in humans were applied to this study, this would suggest that the observed blood pressure increase may not be sufficiently large enough to be considered clinically hypertensive. Therefore, although the 300 mGy female mice were hypertensive by definition of comparison to sham-irradiated controls, this may not be a pathologically relevant increase in BP. Similarly, there was only a ~6% decrease in HR at 1000 mGy in female pups, in the same range as the BP response at 300 mGy, although it is difficult to extrapolate this to a similar pathological increase in humans. It can be noted that larger magnitude changes of HR are possible in mice, such as Wikström et al. (1998) who reported a decrease in 24-hour mean HR from 632 bpm in wildtype animals to 515 bpm (a ~18.5% decrease) in a TRa1 thyroid hormone receptor knockout mouse line. This would suggest

that the 6% decrease in HR observed in this study may not sufficiently large to be of clinical relevance although it is important to emphasize that conclusions about the clinical relevance are difficult to make without relevant values for cardiovascular measures in mice. Collectively, the BP and HR responses might suggest the lack of a pathological phenotype in the offspring, despite a statistically significant change in these parameters.

Although not directly quantified, changes in HR and BP may have implications for other cardiovascular measures including stroke volume (SV) and total peripheral resistance (TPR) in the mice. Cardiac output (CO) is the product of HR and SV and is the rate at which the ventricles of the heart pumps blood. To maintain CO, an increased HR in the transported sham mice would suggest that SV was correspondingly decreased. Similarly, CO is also the ratio of MAP divided by TPR (Lund-Johansen, 1980). Therefore, if CO was maintained, the increased MAP in transported sham mice may have also increased TPR. Measurement of these or other haemodynamic parameters may provide further insights into the exact cardiovascular phenotype of the animal.

3.4 Conclusions and Future Directions

This thesis provided the first investigation into the role of *in-utero* exposure to ionizing radiation and effects on postnatal cardiovascular physiological measures of BP and HR and offspring growth. Habituation of animals to the irradiation protocol was done in an attempt to acclimate pregnant mothers to the transportation and prevent unintended programming of offspring due to stress caused by transportation. Despite this, there was evidence that this was not completely prevented, with transportation stress evident in

male pups. Remarkably, a single dose of 10 mGy radiation to the mother at gestational day 15 appeared to have attenuated the transport effects.

Due to the confounding transportation stress, it was difficult to directly study the role of prenatal irradiation on developmental programming. Therefore, modifications in the irradiation protocol are necessary for future studies on the effects of low dose prenatal irradiation without a confounding variable of transportation stress. The repetitive nature of the transportation of animal cages appeared to subject the mothers to chronic stress during pregnancy and may have resulted in the reported programming. Therefore, limiting transportation of pregnant mothers to only the day of irradiation may be a necessary modification for further experiments. However, a single transport approach would likely increase acute stress from the travel on the day of irradiation and require greater acclimation in the irradiation facility following transportation but prior to irradiation (for example for one hour rather than the 20 minutes used in this thesis). A shift towards a single transportation of the pregnant animal, with a longer acclimation prior to irradiation, would significantly decrease the number of animals that may be irradiated on a given day. One disadvantage to this modification would be the opportunity for greater variation in results due to uncontrollable day-to-day variations (for example weather conditions).

As discussed in Chapter 1, predicting pathological outcomes of gestational exposure to ionizing radiation depends on factors other than total absorbed dose. For example, irradiating animals at a higher dose rate may provide a stronger programming effect due to the known dose-rate effect (Hall & Giaccia, 2012). This is an important consideration

for future studies, due to the lack of a robust and consistent programming phenotype with prenatal irradiation in this study. The magnitude of the cardiovascular changes was relatively small, and a stronger stressor during pregnancy may be necessary.

The strain of mouse used for these studies is an important consideration. The C57Bl mouse strain is a widely used mouse strain in numerous areas of biological research. The availability of transgenic and knockout lines in a C57Bl background makes it an appealing strain of choice for study. However, the high rates of cannibalism that were experienced in this study, which limited the number of pups available per mother, increased the numbers of mothers needed to be irradiated, and prevented the collection of birth weight, were a major limitation and may be resolved the use of an alternative strain of mouse. This incidence of high cannibalism rates may have been due to a combination of poor pup quality at birth due to the stress caused by the transportation of pregnant mothers and that C57Bl mothers are relatively poor mothers. For example, when comparing maternal behaviour between C57Bl/6J and DBA/2J strains, C57Bl/6J females spent less time engaged in maternal behaviours and had a lower survival of the first litter pups (Brown et al., 1999). Furthermore, female C57Bl mice have been reported to be a more relatively radioresistant mouse strain compared to the BALB/c strain with respect to the induction of mammary cancer by whole-body irradiation (Ullrich et al., 1996). The use of a more radiosensitive strain in future studies, with better maternal behaviours and reasonable litter sizes, may be necessary to produce an offspring with a pronounced hypertensive phenotype due to prenatal irradiation.

There is growing experimental evidence supporting the role of oxidative stress in developmental programming, which may present a candidate mechanism at the cellular level for the observed responses in this thesis (Franco et al., 2002). Indirectly ionizing radiation (such as gamma rays produced by the ^{137}Cs source used in this thesis) primarily interacts through molecules within the cell, such as water, to form reactive free radical species that further interact with target molecules such as DNA (Hall & Giaccia, 2012). Prenatal exposure to high doses of ionizing radiation and subsequent oxidative damage at the cellular level presents a possible mechanistic link between prenatal exposure to ionizing radiation and subsequent programming of offspring (Azzam et al., 2012). It is difficult to directly measure oxidative stress, however assays that involve quantification of 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidized derivative of deoxyguanosine may provide further insight into the oxidative state at the cellular level (Valavanidis et al., 2009). Gene expression within tissue samples from pups may also be an area of future research as it has been proposed that the mechanism of oxidative stress programming may be through a direct regulation of gene expression (Luo et al., 2006). Identification of the precise mechanism of this response is important to better understand the role of ionizing radiation exposure during prenatal development.

3.5 References

Alexander, B.T., 2006. Fetal programming of hypertension. AJP: Regulatory, Integrative and Comparative Physiology 290, R1–R10.

Azzam, E.I., Jay-Gerin, J.-P., Pain, D., 2012. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Letters 327, 48–60.

Brown, R.E., Mathieson, W.B., Stapleton, J., Neumann, P.E., 1999. Maternal behavior in female C57BL/6J and DBA/2J inbred mice. Physiol. Behav. 67, 599–605.

Cianfarani, S., Germani, D., Branca, F., 1999. Low birthweight and adult insulin resistance: the "catch-up growth" hypothesis. Archives of Disease in Childhood - Fetal and Neonatal Edition 81, F71–F73.

Franco, M. do C.P., Dantas, A.P.V., Akamine, E.H., Kawamoto, E.M., Fortes, Z.B., Scavone, C., Tostes, R.C.A., Carvalho, M.H.C., Nigro, D., 2002. Enhanced oxidative stress as a potential mechanism underlying the programming of hypertension in utero. J. Cardiovasc. Pharmacol. 40, 501–509.

Godfrey, K.M., Barker, D.J., 2001. Fetal programming and adult health. Public Health Nutrition 4.

Hales, C.N., Barker, D.J., 1992. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 35, 595–601.

Hales, C.N., Desai, M., Ozanne, S.E., 1997. The Thrifty Phenotype Hypothesis: How Does it Look After 5 Years? Diabetic Medicine 14, 189–195.

Hall, E.J., Giaccia, A.J., 2012. Radiobiology for the radiologist. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Holemans, K., Aerts, L., Van Assche, F.A., 2003. Fetal Growth Restriction and Consequences for the Offspring in Animal Models. Reproductive Sciences 10, 392–399. doi:10.1016/S1071-55760300134-5

Jensh, R.P., Eisenman, L.M., Brent, R.L., 1995. Postnatal Neurophysiologic Effects of Prenatal X-irradiation. International Journal of Radiation Biology 67, 217–227.

Kapoor, A., Dunn, E., Kostaki, A., Andrews, M.H., Matthews, S.G., 2006. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids: Fetal programming of HPA function. The Journal of Physiology 572, 31–44.

Langley-Evans, S.C., 1996. Intrauterine programming of hypertension in the rat: Nutrient interactions. Comparative Biochemistry and Physiology Part A: Physiology 114, 327–333.

Li, Y., Gonzalez, P., Zhang, L., 2012. Fetal stress and programming of hypoxic/ischemic-sensitive phenotype in the neonatal brain: Mechanisms and possible interventions. Progress in Neurobiology 98, 145–165.

Lund-Johansen, P., 1980. Haemodynamics in Essential Hypertension. Clinical Science 59, 343s–354s.

Manning, J., Vehaskari, V.M., 2001. Low birth weight-associated adult hypertension in the rat. Pediatric Nephrology 16, 417–422.

Maulik, D., Frances Evans, J., Ragolia, L., 2006. Fetal growth restriction: pathogenic mechanisms. Clin Obstet Gynecol 49, 219–227.

Minamisawa, T., Hirokaga, K., 1996. Long-Term Changes in Open Field Activity of Male Mice Irradiated with Low Levels of Gamma Rays at Late Stage of Development. Journal of Radiation Research 37, 117–124.

Minamisawa, T., Hirokaga, K., 1995. Long-Term Effects of Prenatal Exposure to Low Levels of Gamma Rays on Open-Field Activity in Male Mice. Radiation Research 144, 237.

Murphree, R.L., Pace, H.B., 1960. The Effects of Prenatal Radiation on Postnatal Development in Rats. Radiation Research 12, 495.

Nash, D.J., Gowen, J.W., 1962. Effects of X-Irradiation Upon Postnatal Growth in the Mouse. Biol Bull 122, 115–136.

Ozaki, T., Nishina, H., Hanson, M.A., Poston, L., 2001. Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. The Journal of Physiology 530, 141–152.

Roberts, J.M., Lain, K.Y., 2002. Recent Insights into the Pathogenesis of Pre-eclampsia. Placenta 23, 359–372.

Robinson, S.C., Brucer, M., 1939. Range of Normal Blood Pressure: A Statistical and Clinical Study of 11,383 Persons. Archives of Internal Medicine 64, 409.

Streffer, C., Molls, M., 1987. Cultures of preimplantation mouse embryos: a model for radiobiological studies. Adv Radiat Biol 13, 169–213.

Ullrich, R.L., Bowles, N.D., Satterfield, L.C., Davis, C.M., 1996. Strain-dependent susceptibility to radiation-induced mammary cancer is a result of differences in epithelial cell sensitivity to transformation. Radiat. Res. 146, 353–355.

United Nations. Scientific Committee on the Effects of Atomic Radiation [UNSCEAR], 1977. Sources and effects of ionizing radiation: 1977 report to the General Assembly, with annexes.

Valavanidis, A., Vlachogianni, T., Fiotakis, C., 2009. 8-hydroxy-2′ -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. Journal of Environmental Science and Health, Part C 27, 120–139.

Wei, J.-N., Sung, F.-C., Li, C.-Y., Chang, C.-H., Lin, R.-S., Lin, C.-C., Chiang, C.-C., Chuang, L.-M., 2003. Low Birth Weight and High Birth Weight Infants Are Both at an Increased Risk to Have Type 2 Diabetes Among Schoolchildren in Taiwan. Diabetes Care 26, 343–348.

Wikstrom, L., 1998. Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1. The EMBO Journal 17, 455–461.