Multiple Ingredient Dietary Supplement and Protective Effects in Gamma Irradiated Mice

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

McMaster University Masters of Science (2015)
1280 Main Street West, Hamilton, Ontario, L8S 4K1
Biology

TITLE: Multiple Ingredient Dietary Supplementation and Protective Effects in Gamma Irradiated Mice

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NUMBER OF PAGES: vii, 92

Abstract

Cognitive impairment, "Chemofog", has been well established as a negative outcome of otherwise successful medical radiation treatments. Mitigation of this negative feature would dramatically increase quality of life for those recovering from cancer treatment. There is currently no known intervention to protect or restore cognitive function of patients undergoing radiation treatments. Development of a multiple ingredient dietary supplement (MDS) is meant to offer a non-invasive therapy to help mitigate risk and decrease damage to individuals. The MDS was originally designed to off-set 5 key mechanisms associated with aging including oxidative damage, inflammation, impaired glucose metabolism, mitochondrial dysfunction and membrane deterioration. Radiation damage shares many of the same deficiencies that develop with age and supplementation with MDS would impact many of the same pathways. Changes in cytokine profile (inflammation markers), and biomarkers of behavioural functions, sensory functions, and oxidative damage provide preliminary evidence of MDS impacts.

Acknowledgments

First thanks must go to my supervisors Dr. C. David Rollo and Dr. Douglas Boreham. These brilliant scientists have offered me tremendous opportunity and support. I would not have been able to develop this project or my skills as a scientist without them. I benefited greatly from their perspective and insight. I must also offer sincere thanks to my additional supervisory committee members Lovaye Kajiura, and Sue Becker. Special recognition is necessary for the help and guidance I received from post-doc Jennifer Lemon as well as Mary-Ellen Cybulski. I must also thank Vadim Askenov for his contribution to data analysis and providing me with the knowledge necessary to run Statistica. Without his guidance I would have been lost.

Sincere thanks to all the students, volunteers and technicians who assisted in animal care, diet preparation, lab work, and data collection. Names and more specific acknowledgements can be found in the declaration of academic achievement.

I am grateful for funding support from the Natural Sciences and Engineering Research Council of Canada and funding support from Dr. C. David Rollo and Dr. Douglas Boreham.

Many thanks must go to my family and friends without whose support this would have not been possible.

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DECLARATION OF ACADEMIC ACHIEVEMENT

All ideas expressed are those of Kathleen Monster unless otherwise referenced. The multiple ingredient dietary supplement was originally invented by C. David Rollo, Douglas Boreham, and Jennifer Lemon (2000-2004).

Mice were supplied by Jackson laboratories. Preparation and administration of MDS was completed by Kathleen Monster, Jennifer Lemon, and student workers Jasmine Singh, Baljit Grewal, and Navjot Khela. Blood samples were collected by Kathleen Monster, Jennifer Lemon and Mary-Ellen Cybulski. The Luminex assay system was provided by Dawn Bowdish and the cytokine multiplex was run with the assistance of Chris Verscheer, Kathleen Monster, Jennifer Lemon, and Lisa Stoa. Novel object recognition and novel placement recognition testing was carried out by Kathleen Monster and Jennifer Lemon. Video assessment of behavioural tasks was performed by Kathleen Monster, Roberta Boreham and Jacob Bates. Statistical analysis was completed by Kathleen Monster. Olfactory testing was completed by Kathleen Monster. DNA/RNA oxidative damage was assessed by Lisa Stoa and Kathleen Monster with guidance from Jennifer Lemon. Analysis of damage was done by Kathleen Monster.

INTRODUCTION

Chemofog.

Chemofog is a pervasive symptom of medical radiation treatment. Symptoms can include difficulty concentrating, impaired memory recall and changes in verbal working memory. These changes are often persistent, lasting for years (Bender et al., 2006). Exact mechanisms causing impairment are uncertain but induction of cognitive impairment from radiation is well established. Memory, learning, and executive functioning are all negatively impacted. Many cancer survivors suffer for years from demoralizing loss of function. Chemofog, a terrible side effect of lifesaving therapy, is not yet preventable or treatable.

Radiation.

Radiation kills through two main mechanisms: direct damage and indirect damage. Direct damage is caused by direct interaction of a ray with a DNA strand. This interaction can cause single strand breaks, double strand breaks, or base damage leading to replication errors. Indirect damage normally works through hydrolysis and free radical formation and propagation leading to oxidation, alkylation or hydrolysis of bases. This can block replication or cause replication errors (Podgorsak, 2005). Radiation damage can induce apoptosis, poorly functioning cells or cancerous growth. If enough cells in a tissue are killed tissue function will be compromised.

Antioxidants and Radiosensitivity.

Augmentation of oxidative stress and the propagation of free radicals is a key mechanism responsible for cell killing in both radiation and chemotherapy treatments

(Dayal, Singh, Pandey, & Mishra, 2014). Oxidative stress (OS) and the production of reactive oxygen species (ROS) is a common mechanism of many pathologies. There are multiple cell targets affected. Enzyme systems are in place to control oxidative status but can be overwhelmed during stress or injury (Slemmer, Shacka, Sweeney, & Weber, 2008). Some ROS, however, are required in order for cells to survive. Homeostasis must be preserved and a delicate balance of ROS is maintained (D'Autréaux & Toledano, 2007; Dayal et al., 2014).

Antioxidants are one of the most effective protectors against oxidative damage. They can greatly reduce oxidative stress. Since a main actor of radiation damage is oxidative stress the logical conclusion is that antioxidants can provide some protection from radiation induced damage. Damage from whole body exposure to radiation at both low and high dose ranges may be reduced. This is a key benefit under accidental exposure scenarios or when used in conjunction with diagnostic imaging. Reduction of ROS may help to improve the therapeutic index of radiation treatments by protecting normal tissue. The question remains; are there detrimental effects linked to these compounds?

During cancer treatment the term "therapeutic index" describes the balance between maximized cell killing in tumours and minimized damage to normal tissue. Supplementation using products that may decrease oxidative stress during cancer treatment should be considered carefully since they may negatively impact cell killing in tumours. Antioxidant status of the cytoplasm is a determining factor of cytotoxic effects in cancer treatments (Franco & Cidlowski, 2009). Interfering with the cells ability to resist oxidative stress may improve treatment outcomes by increasing tumour cell death.

Ideally, a treatment that would increase oxidative stress in tumours while decreasing oxidative status in normal tissue would promote cell killing in the tumour and protect normal tissue. This balance would increase the therapeutic index and be greatly beneficial to treatment outcomes.

Antioxidants, both endogenous and dietary, can decrease tumour response to radiation treatment. Antioxidants have been proven to also protect normal tissue. Therefore administration of therapeutic level (high) doses of antioxidant can potentially improve therapeutic indices (Block et al., 2007; Prasad, Kumar, Kochupillai, & Cole, 1999). The therapeutic mix, "triphala", as an example, has been identified as an antioxidant and increases apoptosis in cancer cells in a dose dependant manner (Sandhya, Lathika, Pandey, & Mishra, 2006).

Antioxidants can also produce negative effects. Normally stressors such as radiation or chemotherapy increase ROS to a level that results in apoptosis. If antioxidant use can reduce ROS levels below the apoptosis threshold, severely damaged cells can live and act pathologically (Sandhya & Mishra, 2006).

Diet Origins and Design.

Dietary supplementation is a field that is fraught with controversy. Production and purification of nutraceuticals can be difficult to regulate which can result in a variable final product. Plant extracts can contain different concentrations of the most biologically active molecules from year to year based purely on variable growing conditions so even with strict control of harvest and processing an inferior product can be produced. Despite these difficulties nutraceuticals have gained favour in the research community as

important factors to increase radiosensitivity and cell killing. Widespread effects of nutraceuticals have not been well quantified and complex mixtures of nutraceuticals are truly in their research infancy (Deorukhkar, Krishnan, Sethi, & Aggarwal, 2007).

Support for individual natural compounds has been established. Melatonin is theorized to prevent saturation of repair pathways. Less saturation means that more damage is fixed via repair pathways instead of apoptosis (Shirazi, Ghobadi, & Ghazi-Khansari, 2007). Curcumin, derived from turmeric, has been shown to activate Nrf2 transcription which leads to downregulated inflammation. Dietary factors such as curcumin have also been shown to inhibit carcinogenesis (Martín-Montalvo, Villalba, Navas, & de Cabo, 2011). Curcumin acts as a NSAID and can reduce levels of oxidized proteins in the cell (Slemmer et al., 2008). Quercetin is a powerful antioxidant with an antioxidant capacity of 3.5 times that of curcumin. It is able to reduce ROS and nitric oxide derivatives

Exact ratios of elements may be central to the efficacy of complex mixtures such as the multiple ingredient supplement used in this study. For example, Vitamin E is absorbed more easily when ingested with fats (Slemmer et al., 2008) meaning that the balance of lipids present in the dietary supplement and the meals with which it is taken may have significant impact on its function.

MDS was originally designed to off-set 5 key mechanisms associated with aging including: oxidative damage, inflammation, impaired glucose metabolism, mitochondrial dysfunction, and membrane deterioration. Thirty ingredients that have shown promise as individual supplement were combined in a proprietary formula to produce a specially

designed MDS (Table 1). Biological effects of ingredients were previously published. Both γH2AX and 8-hydroxy-deoxyguanosine were reduced by MDS. This diet formulation reduced chromosomal aberrations after 2Gy whole body γ ray exposure (J. A. Lemon, Rollo, & Boreham, 2008a).

Goals of Study.

The goal of this study is to determine if a multiple ingredient dietary supplement can provide protection from radiation induced damage. Treatment with our specially developed MDS may offer improvement of the therapeutic index through both increased tumour cell killing and protection of normal tissue. Many different resistance mechanisms modifying cell metabolism are responsible for radioresistance to oxidative stress. This study aims to look at the protective effects of the MDS and possible ability to mitigate damage impact after injury.

Major Study Design Features.

In the current study two mouse models were used to study the radiation effects on cognitive function. Phase one mice, C57BL/6J, were divided into 5 groups including a control group, MDS only group, irradiation only group a group fed MDS continuously before and after irradiation and a group fed MDS only post-irradiation. A whole body irradiation dose of 5Gy was used. This dose maximizes damage without mortality. Phase two mice, TG (Thy1-EGFP) MJrs/J, mice with brain-only expression of green fluorescent protein were divided into the same groups. A head only dose of 10Gy was used. A 10Gy whole body dose would be fatal, but a higher dose allows for more substantial damage impacts on neuronal physiology and function. A scatter dose group of 0.5Gy was added to

help distinguish abscopal effects from the now more substantial non-targeted body dose (Table 2). Body dosimeters were used to determine the scatter dose. TGF mice are bred on a C57BL/6J background and are functionally very similar to C57 mice. Green fluorescent proteins were added in phase two mice in order to facilitate clear neuronal imaging during further analysis.

Statistics.

All statistical testing described throughout this thesis were performed using Statistica 10, StatSoft Inc. Analysis of Variance was performed on all data sets and post-hoc analysis using Duncan's multiple range test using a criterion of $p<0.5$ determined significance.

Tables

Table 1: Nutraceuticals and dosages included in the Multiple Ingredient Dietary Supplement.

Component	Daily Dose /1 Mouse (mg)
B1	0.72
B3 (Niacin)	0.72
B6	0.72
B12	0.00072
C	3.6
D (1000IU = 0.025mg)	0.0000625
Acetyl L-Carnitine	14.4
Alpha Lipoic Acid	0.72
ASA	2.5
Beta Carotene (30000IU = 18mg)	0.03
Bioflavinoids	4.32
Chromium Picolinate	0.00144
CoEnzyme Q10	0.44
Curcumin	1.8
Folic Acid	0.01
Garlic (allicin)	0.0216
Ginger	7.2
Gingko Biloba	1.44
Ginseng	8.64
Green Tea	7.2
L-Glutathione	0.36
Magnesium	0.72
Melatonin	0.01
N-Acetyl Cystein	7.2
Potassium	0.36
Quercitin	0.9
Rutin	0.72
Selenium	0.00108
TOTAL:	
Oils	
E (400IU = 268mg)	0.965
Omega 3/6/9	21.6

Table 2: Phase one and phase two treatment groups.

Phase One C57 Mice	Phase Two GFP Mice
1) Control	1) Control
2) MDS Only	2) MDS only
3) 5 Gy Whole body irradiation	3) 10Gy Brain irradiation only
4) MDS Pre and Post-Irradiation	4) MDS Pre and Post-Irradiation
5) MDS Post-Irradiation	5) MDS Post-Irradiation
	6) Scatter (0.5 Gy)

CHAPTER 1:

IMPACTS OF MDS ON INFLAMMATION PROFILE OF GAMMA IRRADIATED MICE

1.1 Introduction

Cycles of inflammation play a central role in the long-term effects and damage caused by radiation. Long-term increases in inflammatory status of atomic bomb survivors has been confirmed to be dose dependant (T. Hayashi et al., 2012) just as increased risk of disease is dose dependant. Atomic bomb survivors have increased risk of cardiovascular disease, cancer, and autoimmune diseases. There is little explanation for such long term changes in disease risk other than low-grade systemic inflammation (T. Hayashi et al., 2005).

In vivo exposure to radiation begins a cytokine cascade that includes pro-inflammatory cytokines TNF-α, IFN, Interleukin (IL)-1β, IL-6 (Schaue, Kachikwu, & McBride, 2012), and anti-inflammatory cytokines IL-4, and IL-10 (Han, Song, Yun, & Yi, 2006). Pro-inflammatory cytokine levels (TNF-α, IFN-γ, IL-1, and IL-6) begin to rise within minutes of irradiation (Schaue et al., 2012). High levels of circulating pro-inflammatory cytokines has been linked to cancer cell invasion and cancer growth. IL-1β and IL-6 have been directly linked to cancer cell invasion and IL-6 has even been linked to distant metastases in breast cancer patients (Dethlefsen, Højfeldt, & Hojman, 2013; Paquette, Therriault, & Wagner, 2013). TFN-α has been linked to cancer initiation but only weakly associated with cancer promotion (Moore et al., 1999).

Persistence of elevated cytokine profiles has been connected to apoptosis and tissue fibrosis. Many chronic progressive diseases have markers of constantly elevated systemic inflammation. For some diseases, progression can be slowed using therapies designed to lower systemic inflammation (Forrester & Bick-Forrester, 2005).

Anti-inflammatory cytokines counteract and control pro-inflammatory cytokine levels after injury and repair. Inflammation can promote damage repair, but persistent, chronic inflammation is heavily linked to multiple pathologies. IL-4 and IL-10 are classic examples of anti-inflammatory cytokines. IL-6 can be classified at both a pro and anti-inflammatory cytokine in specific situations but is generally considered a key pro-inflammatory cytokine in systemic chronic inflammation.

Cycles of hypersensitivity to pain can be set up and maintained or those cycles can be destroyed based on the balance of inflammatory and anti-inflammatory cytokines (Zhang & An, 2007). The eventual decline in inflammation is in part because of the short half-life of cytokines and in part because of shifting balance between pro-inflammatory and anti-inflammatory cytokines levels. Anti-inflammatory levels are increased in relation to pro-inflammatory cytokines arrest the cycle of inflammation (Sultani, Stringer, Bowen, & Gibson, 2012).

Cytokines act as an intricate system and a complex-web of interaction between cytokines means that it is very difficult to determine any cause and effect relationships between outside stimuli and isolated cytokine levels. Exact cytokine profiles, after irradiation, vary greatly due to dose, tissue irradiated and individual radiation sensitivity. Polymorphisms in genes affecting cytokine distribution are not rare and can be the cause

of considerable individual variation (Venkatesh et al., 2014). Inhibition of the inflammatory cytokine cascade or the late cycles of low-grade chronic inflammation would be ground breaking for the treatment of many diseases.

Cytokine Control.

Cytokines are produced in a complex cascade. Each cytokine impact the others and a balance of cytokines is always present. Stressors can change the equilibrium and negatively impact health. Many cytokines share feedback cycles that tie their production together.

Interleukin-6. IL-6 is considered a primary marker for inflammation status and is detectable in atomic bomb survivors over 60 years post exposure (T. Hayashi et al., 2012). IL-6 has been linked to radiation resistance and inhibition of this cytokine tended to increase radiation sensitivity (as seen in prostate cancer cells) (Wu, Chen, Chen, & Hsieh, 2013). IL-6 is involved in nerve regeneration and the activation of microglia and astrocytes. IL-6 is also an upstream regulator of neuropeptide expression (Zhang & An, 2007). IL-6 is involved in the transition from an acute inflammation state towards a more balanced cytokine load. This cytokine initiates production of TNF-α and IL-1 antagonists.

IL-6 can activate both STAT1 and STAT3. STAT3 is an oncogene that promotes cell proliferation and survival (Shen, Devgan, Darnell, & Bromberg, 2001), but STAT1 increases inflammation responses and increases immunity responses in tumour cells (Calò et al., 2003). In normal mammalian cells STAT activation is transient lasting perhaps a few hours. STAT3 activation in conjunction with IL-6 can upregulate other pro-inflammatory genes including those controlling IL-1β (Samavati et al., 2009).

Nuclear Factor kappa B. Pro-inflammatory cytokines TNF-α, IL-1, IL-2, and IL-6 are controlled by a common pathway involving Nuclear Factor kappa B (NF-kB). NF-kB is part of a pro-survival pathway activated by irradiation in mammalian cells(Maggio et al., 2015). NF-kB suppresses apoptosis and has been shown to increase radioresistance (Aravindan et al., 2014). NF-kB response has been shown to be dose dependant (Rho, Kim, & Lee, 2005). The action of TNF-α, IL-1β, and IL-6 are implicated in pathological pain responses (Zhang & An, 2007). Since they are part of a common pathway, it makes sense for these cytokines to generally vary in expression together.

Several previous studies have explored selectively inhibiting NF-kB or STAT pro-inflammatory pathways. It has been found that inhibition of these pathways could increase radiosensitivity and be used in conjunction with radiation or chemotherapy to increase therapeutic indices and treatment outcomes (Starenki, Namba, Saenko, Ohtsuru, & Yamashita, 2004; Yu, Pardoll, & Jove, 2009).

Interleukin-1β. IL-1β is directly influenced by radiation and activates key inflammation pathways. This cytokine activates matrix metalloproteinases, enzymes that regulate extracellular matrix molecules (W. Liu et al., 2006). IL-1β expression is upregulated following damage to the central nervous system (microglia and astrocytes). Not only do IL-1β levels increase, but levels of IL-1β receptors also increase. This rapid increase in expression indicates a possible role in early acute inflammatory reactions. Increased cytokine levels are not limited only to the site of injury and appear bilaterally in the brain (Copray et al., 2001).

Interleukin-10. IL-10 represses macrophage production of TNF-α, IL-1, and IL-6. IL-10 can also down-regulate pro-inflammatory cytokine receptors (Zhang & An, 2007). IL-10 and IL-4 are possibly key players in chronic non-localized pain since low levels of these cytokines have been present in chronic pain sufferers (Uçeyler et al., 2006). IL-10 can act on occasion as a immunosuppressant, which can allow tumour cells to avoid cell killing (Hamidullah, Changkija, & Konwar, 2011).

1.2 Methods

Animals.

Two strains of male mice were kept, C57Bl/6J and Tg (Thy1-EGFP) MJrs/J mice (Thy1-GFP). Thy1-GFP mice are on a background of C57 mice and have a green fluorescent protein expressed in specific brain regions. All care was provided as outlined in the Animal Utilization Protocol as approved by the Animal Research Ethics Board. Mice were fed and watered *ad libitum* and housed individually. They were exposed to a 12hr/12hr day/night photoperiod.

Feeding.

The complex dietary supplement contained 30 different compounds available over the counter. Doses were determined by human recommended dosages, adjusted for weight, and increased by 10 fold in order to account for the higher metabolic rate of mice. MDS was mixed fresh each week and refrigerated or frozen for short periods of time. Individual doses were administered daily soaked into bagel pieces. Bagel bits were highly favoured and consumed completely. Any partial consumption during habituation periods was recorded daily. Since mice are neophobic towards offered food, all groups that would

be fed MDS at any point received several days of diet habituation at the beginning of the month pre-irradiation. Bi-weekly body weights were recorded to track growth and weight gain during maturation. Food was weighed when topped up in order to provide a consumption record and ensure that there was no unintentional diet restriction or subsequent weight loss.

Groups.

Mice were randomly assigned upon arrival to one of the five groups. MDS was received during specific time interval pre and/or post irradiation. Groups consisted of 5 age matched male mice. Each mouse was sampled only once. The study was initiated with mice between 9 weeks and 11 weeks old. Irradiation occurred 1 month after the initiation of the study.

Irradiation.

All groups not requiring irradiation underwent sham irradiation. Gamma irradiation using a cesium source was used and phase one mice were immobilized for the duration using plastic tubes that allowed for free movement. Phase two mice were immobilized by plastic cones that allowed for body rotation around the coronal/sagittal planes but not the transverse plane. Custom build lead shielding was used to protect the body from irradiation.

Panel.

Serum was separated from collected blood and flash frozen until analysis. The Luminex multiplexing protein array system was used to analyze dilute blood serum.

Luminex assays use flow cytometry, lasers and multi-anlyate profiling beads to determine expression levels of cytokines in the blood (ThermoFisher Scientific, n.d.)

Statistics.

Analyses of Variance were performed and significance between groups was further analyzed using Duncan's multiple range test and the criterion for significance used was $p<0.05$. Effects of time point and group were significant. Differences between strains at the 30 day time point were insignificant therefore control and MDS only groups were pooled. Group size ranged from n=4 (10Gy+MDS) to n=10 (control, MDS at 30days). Most groups contained n=5.

1.3 Results

Cytokine Expression at 2 days (Phase One).

Levels of circulating cytokines were measured in pg/ml at 2, 30 and 120 days. On day 2 several patterns were visible. TNF-α (Fig. 1) and IL-6 (Fig. 2) both have low level expressions of 5Gy and MDS+5Gy+MDS that are significantly lower than Control and 5Gy+MDS levels. For cytokine IL-6 expression 5Gy also expressed higher IL-6 levels than MDS and MDS+5Gy+MDS and lower than 5Gy+MDS.

IFN-γ (Fig. 3), IL-2 (Fig. 4), and IL-4 (Fig. 5) expressed very similar patterns. IFN-γ had group MDS+5Gy+MDS expressing significantly lower levels of cytokine then control and 5Gy+MDS. IL-2 had low level expressions of 5Gy and MDS+5Gy+MDS that are lower than Control, 5Gy+MDS and additionally MDS levels. IL-4 followed the same pattern with low expressions of 5Gy and MDS+5Gy+MDS while MDS+5Gy+MDS was lower than all other groups and 5Gy was lower than MDS and 5Gy+MDS.

IL-12p70 (Fig. 6) had low expression in the MDS group significantly lower than control and MDS+5Gy+MDS group. Two cytokines, IL-1β (Fig. 7) and IL-10 (Fig. 8), had very high levels of expression in the 5Gy+MDS group. IL-1β was expressed in the 5Gy+MDS group higher than Control, MDS, and MDS+5Gy+MDS. IL-10 was expressed in the 5Gy+MDS group higher than all other groups.

Cytokine Expression at 30 days (Phase One).

On day 30, a solid pattern emerged. TNF-α (Fig. 9), IFN-γ (Fig. 10), IL-1β (Fig. 11), IL-6 (Fig. 12), IL-2 (Fig. 13), IL-4 (Fig. 14) and IL-10 (Fig. 15) all displayed a similar pattern with very low levels of cytokine expressed in the 5Gy group and statistically similar low levels of expression in the 5Gy+MDS group. The levels of 5Gy+MDS were not necessarily significantly different from other groups. Expression levels in MDS and MDS+5Gy+MDS groups for these cytokines remained generally higher than the 5Gy group except for IL-2 and IL-10 where group MDS+5Gy+MDS was not statistically different from 5Gy. Levels of expression in control groups were statistically higher than 5Gy except in IL-10. Cytokine expression in groups 5Gy+MDS was statistically lower for IFN-γ, IL-1β, IL-6, IL-2 and IL-4. The cytokines IL-10 still had a difference between 5Gy+MDS and MDS. TNF-α had no differences between 5Gy+MDS and other groups.

Cytokine IL-12p70 (Fig. 16) did not fit the pattern shown by the other cytokines. The 5Gy group was still statistically different than the Control, MDS and 5Gy+MDS but expression levels were highest in 5Gy.

Cytokine Expression at 30 days (Phase Two).

Phase two mice underwent higher doses of radiation. Pro-inflammatory cytokines IFN-γ (Fig. 17), IL-1β (Fig. 18), and IL-6 (Fig. 19) all displayed a similar pattern with very low expression in the control group compared to the 10Gy group. Levels of IFN-γ expression in the 10Gy+MDS group were lower than all other groups. Levels of IL-1β in the control group were lower than all the radiation involved groups (10Gy, Scatter, and MDS+10Gy+MDS) except 10Gy+MDS. In addition to the low levels of expression in the control group, IL-6 had low levels of expression in the MDS group compared to the 10Gy group.

Anti-inflammatory cytokines IL-2 (Fig. 20), IL-10 (Fig. 21) followed a similar pattern as the pro-inflammatory cytokines IFN-γ, IL-1β, and IL-6 with very low levels of cytokine expression in the control group compared to the 10Gy group. IL-2 had expression higher in the 10Gy group than the Control, MDS and 10Gy+MDS groups. IL-10 followed the same pattern as IL-1β with levels of IL-10 in the control group lower than all the radiation involved groups (10Gy, Scatter, and MDS+10Gy+MDS) except 10Gy+MDS. The 10Gy+MDS group had much lower IL-10 expression than the MDS+10Gy+MDS group. IL-4 (Fig 22) appeared to have a similar pattern as cytokine 2 but the difference between the control group and 10Gy did not reach significance. The 10Gy group was significantly higher than the 10Gy+MDS group. Cytokines TFN-α (Appendix A) and IL-12p70 (Appendix A) failed to reach significance.

Cytokine Expression at 120 days (Phase one).

By the 120 day time point cytokine expression had leveled off between many groups. Cytokine expression in the 5Gy group had spiked for TNF-α (Fig. 23) and IFN-γ (Fig. 24) and displayed a pattern similar to IL-12p70 at 30 days. A pattern of low expression in group MDS+5Gy+MDS appears. Both cytokines TNF-α, and IFN-γ had group MDS+5Gy+MDS cytokine expression lower than 5Gy group. IL-6 (Fig. 25) expression in group MDS+5Gy+MDS is significantly lower than the control. In IL-4 (Fig. 26) MDS+5Gy+MDS is lower than the control and 5Gy+MDS. In IL-1β (Fig. 27) MDS+5Gy+MDS is higher than MDS and 5Gy+MDS. Expression levels of IL-2, IL-2 and IL-12p70 (Appendix A) did not reach significance.

1.4 Figures

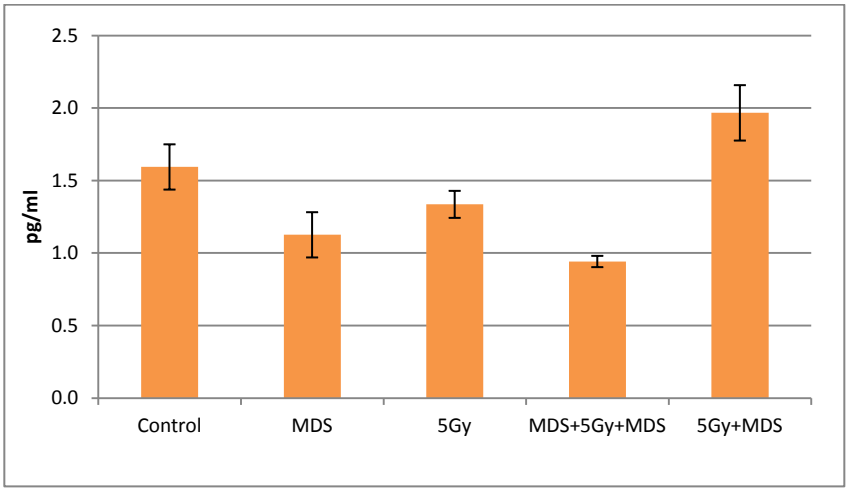

Figure 1: Effects of various treatments on circulating levels of TNF-α in pg/ml at 2 days. Duncan's multiple range test indicated a difference between Control and MDS ($p<0.03$), and MDS+5Gy+MDS ($p<0.004$). Duncan's test also indicated a difference between 5Gy+MDS and MDS ($p<0.0004$), 5Gy ($p<0.004$), and MDS+5Gy+MDS ($p<0.00007$).

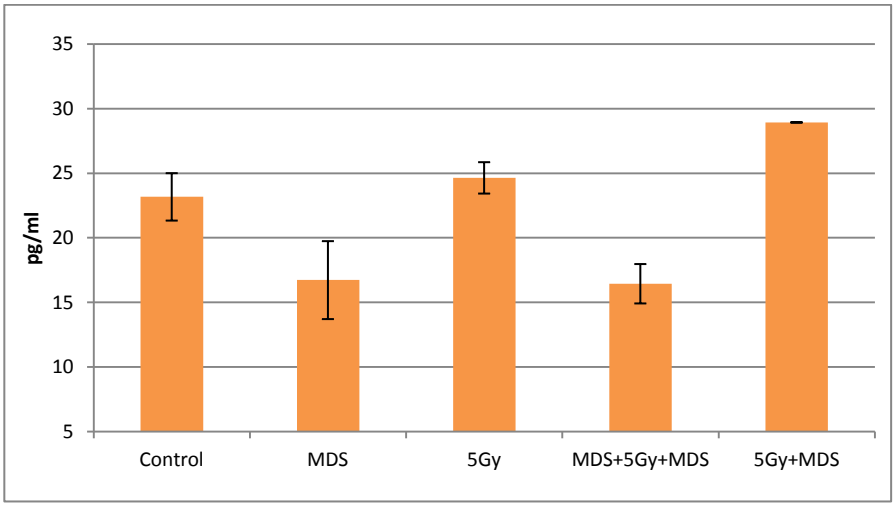

Figure 2: Effects of various treatments on circulating levels of IL-6 in pg/ml at 2 days. Duncan's multiple range test indicated a difference between Control and MDS ($p<0.03$), MDS+5Gy+MDS ($p<0.03$), 5Gy+MDS ($p<0.05$). MDS was different from 5Gy ($p<0.009$) and 5Gy+MDS ($p<0.0004$). 5Gy was different from 5Gy+MDS ($p<0.009$). MDS+5Gy+MDS was different from 5Gy+MDS ($p<0.0003$).

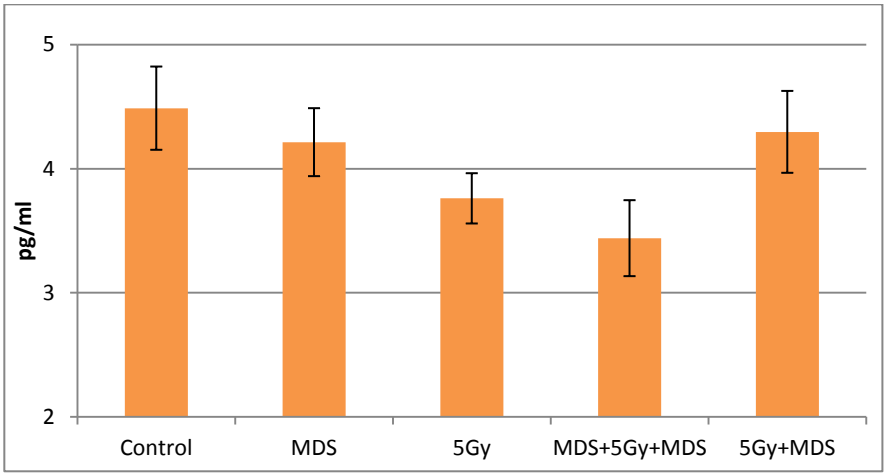

Figure 3: Effects of various treatments on circulating levels of IFN-γ in pg/ml at 2 days. Duncan's multiple range test indicated a difference between MDS+5Gy+MDS and Control ($p<0.02$), and 5Gy+MDS ($p<0.05$).

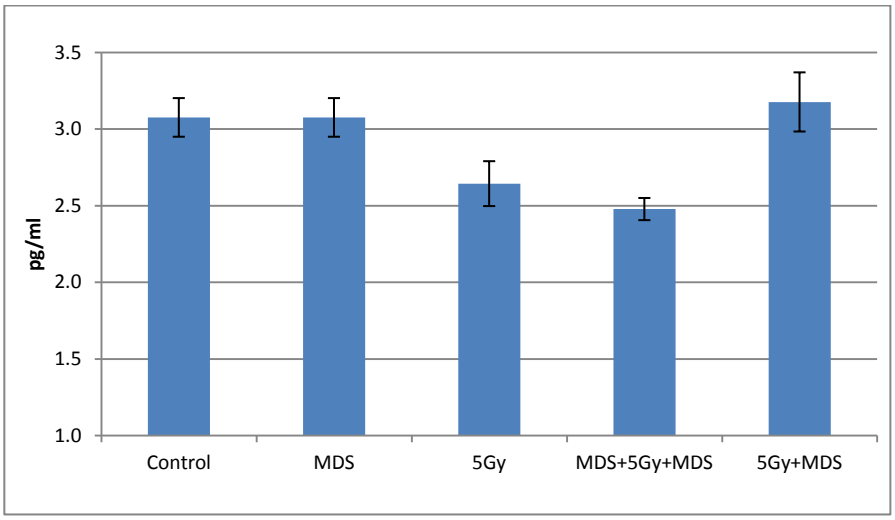

Figure 4: Effects of various treatments on circulating levels of IL-2 in pg/ml at 2 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and 5Gy ($p<0.005$), and MDS+5Gy+MDS ($p<0.006$). Duncan's test also indicated a difference between the 5Gy group and Control ($p<0.02$), MDS ($p<0.02$), and there was also a difference between MDS+5Gy+MDS and Control ($p<0.002$) and MDS ($p<0.002$).

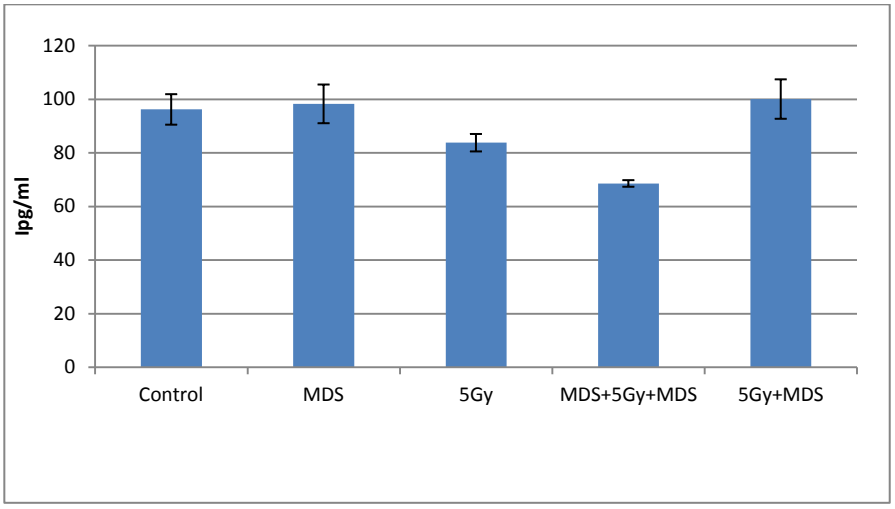

Figure 5: Effects of various treatments on circulating levels of IL-4 in pg/ml at 2 days. Duncan's multiple range test indicated a difference between MDS+5Gy+MDS and Control ($p<0.0007$), MDS ($p<0.0004$), 5Gy ($p<0.04$) and 5Gy+MDS ($p<0.0003$). 5Gy+MDS is also different from 5Gy ($p<0.04$). The 5Gy group is also different than MDS ($p<0.05$).

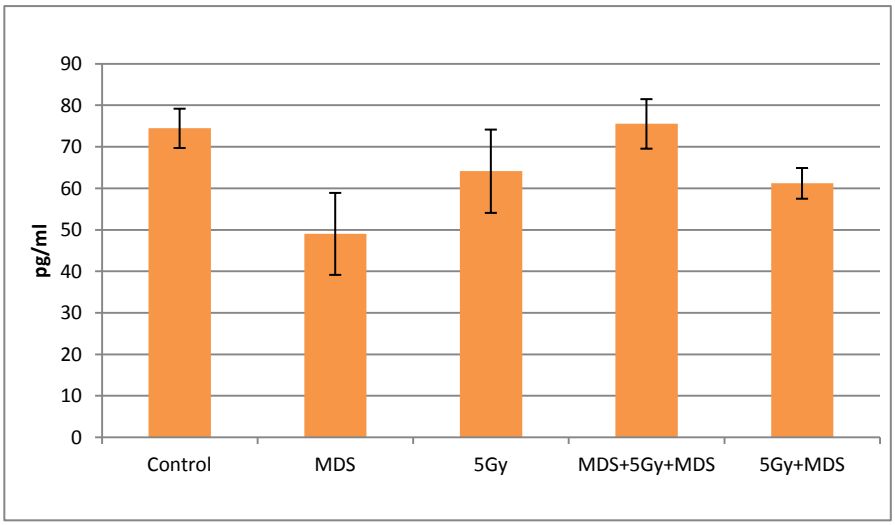

Figure 6: Effects of various treatments on circulating levels of IL-12p70 in pg/ml at 2 days. Duncan's multiple range test indicated a difference between MDS and Control ($p<0.04$), and MDS+5Gy+MDS ($p<0.04$).

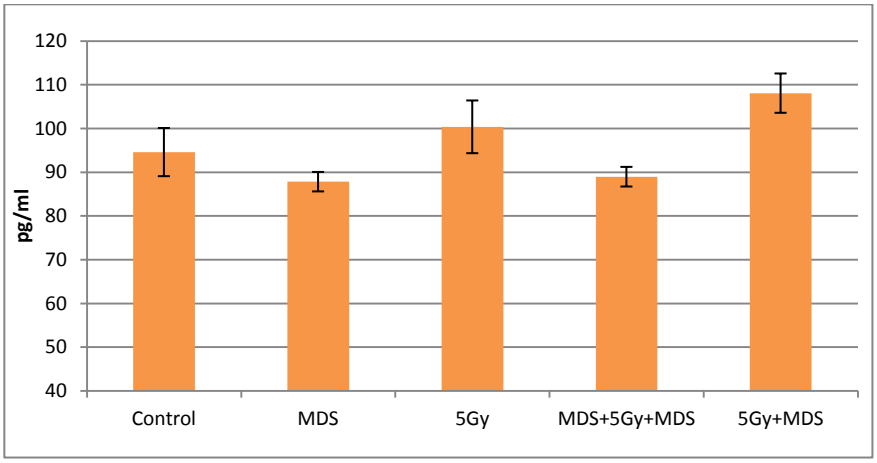

Figure 7: Effects of various treatments on circulating levels of IL-1β in pg/ml at 2 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.04$), MDS ($p<0.004$), and MDS+5Gy+MDS ($p<0.006$).

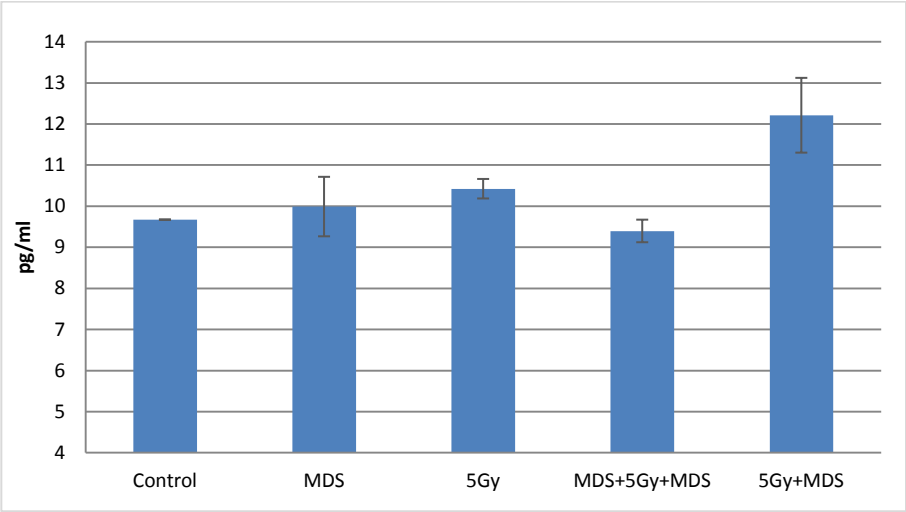

Figure 8: Effects of various treatments on circulating levels of IL-10 in pg/ml at 2 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.001$), MDS ($p<0.003$), 5Gy ($p<0.01$) and MDS+5Gy+MDS ($p<0.0005$).

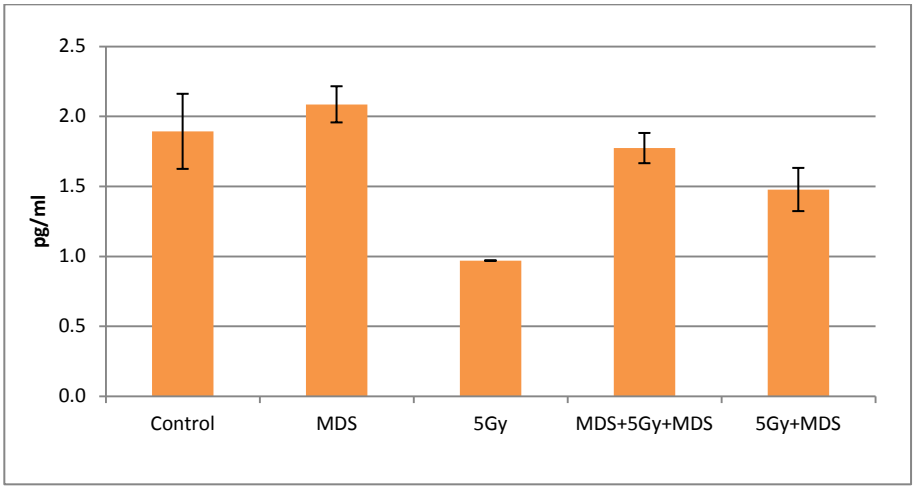

Figure 9: Effects of various treatments on circulating levels of TNF-α in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy and Control ($p<0.009$), MDS ($p<0.002$), and MDS+5Gy+MDS ($p<0.02$).

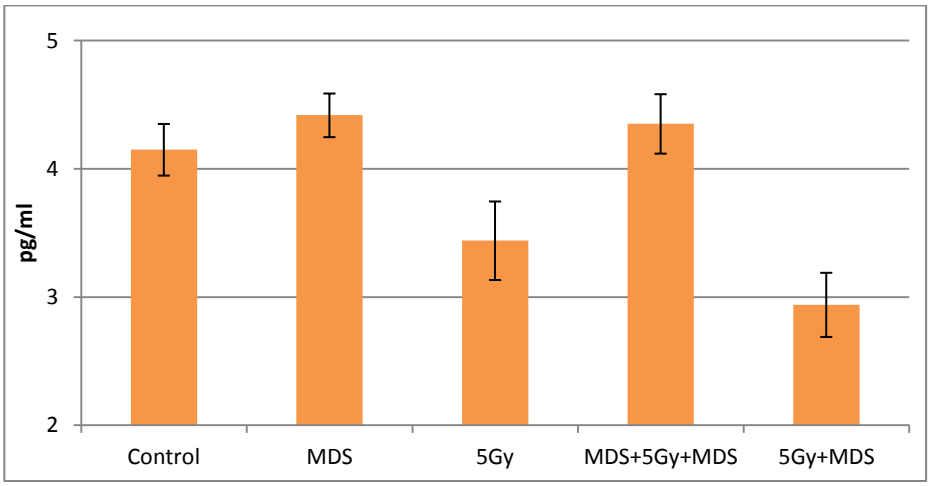

Figure 10: Effects of various treatments on circulating levels of IFN-γ in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.002$), MDS ($p<0.0003$), and MDS+5Gy+MDS ($p<0.0005$). Duncan's test also indicated a difference between the 5Gy group and Control ($p<0.05$), MDS ($p<0.02$), and MDS+5Gy+MDS ($p<0.02$).

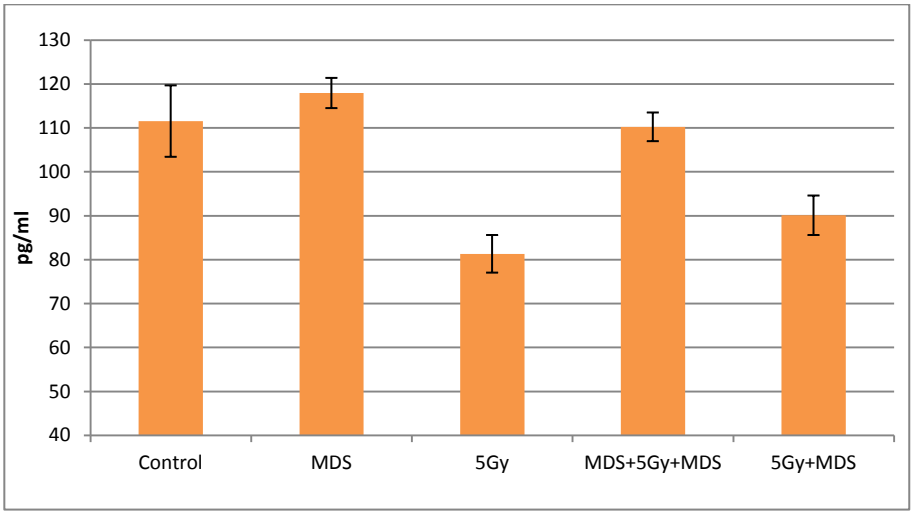

Figure 11: Effects of various treatments on circulating levels of IL-1β in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.04$), MDS ($p<0.009$), and MDS+5Gy+MDS ($p<0.04$). Duncan's test also indicated a difference between the 5Gy group and Control ($p<0.005$), MDS ($p<0.001$), and MDS+5Gy+MDS ($p<0.006$).

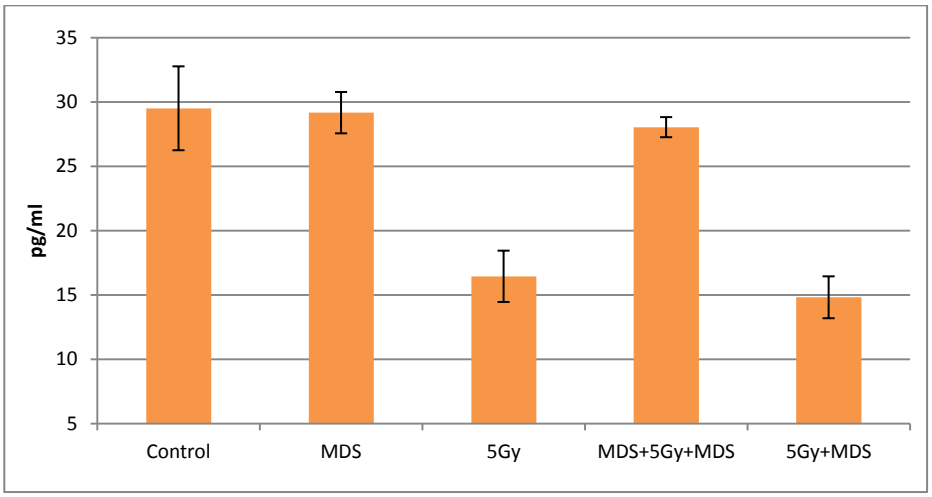

Figure 12: Effects of various treatments on circulating levels of IL-6 in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.002$), MDS ($p<0.002$), and MDS+5Gy+MDS ($p<0.003$). Duncan's test also indicated a difference between the 5Gy group and Control ($p<0.003$), MDS ($p<0.003$), and MDS+5Gy+MDS ($p<0.005$).

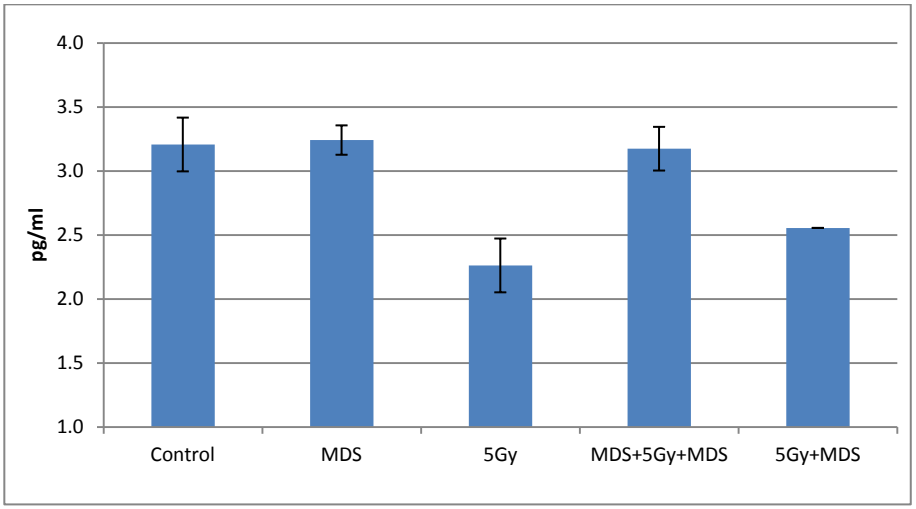

Figure 13: Effects of various treatments on circulating levels of IL-2 in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.03$), MDS ($p<0.03$), and MDS+5Gy+MDS ($p<0.03$). Duncan's test also indicated a difference between the 5Gy group and Control ($p<0.003$) and MDS ($p<0.002$). There was also a difference between MDS+5Gy+MDS and MDS ($p<0.002$).

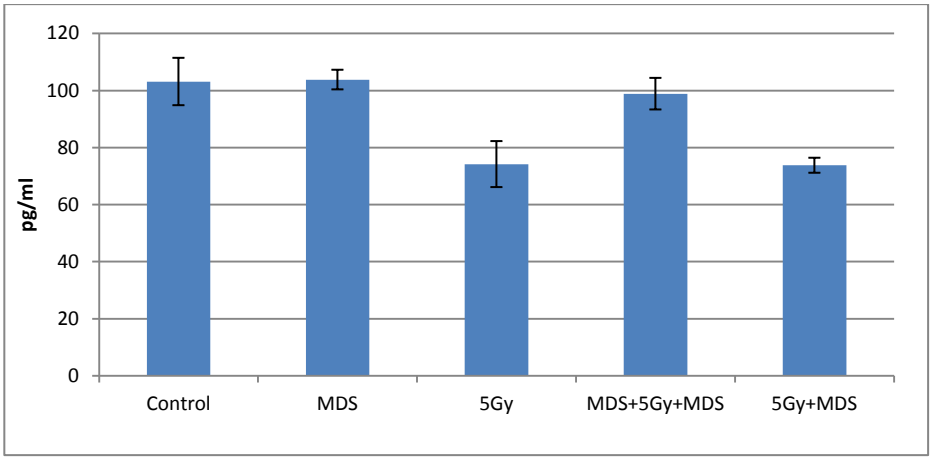

Figure 14: Effects of various treatments on circulating levels of IL-4 in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy+MDS and Control ($p<0.01$), MDS ($p<0.01$), and MDS+5Gy+MDS ($p<0.02$). Duncan's test also indicated a difference between the 5Gy group and Control ($p<0.01$), MDS ($p<0.01$), and MDS+5Gy+MDS ($p<0.02$).

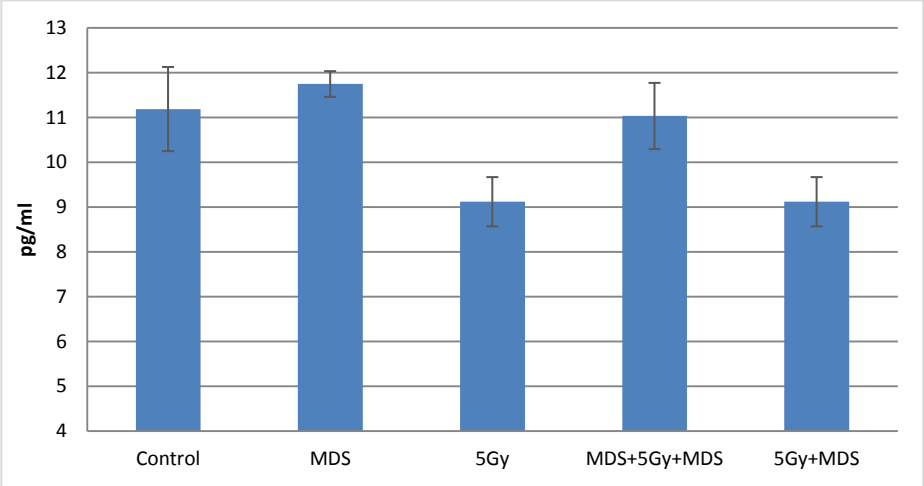

Figure 15: Effects of various treatments on circulating levels of IL-10 in pg/ml at 30 days. Duncan's multiple range test indicated a difference between MDS and 5Gy+MDS ($p<0.04$) and 5Gy ($p<0.04$).

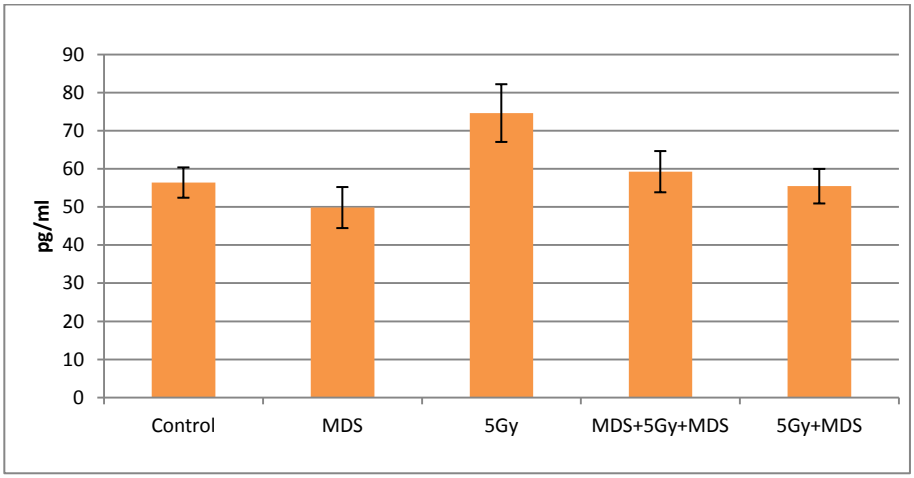

Figure 16: Effects of various treatments on circulating levels of IL-12p70 in pg/ml at 30 days. Duncan's multiple range test indicated a difference between 5Gy and Control ($p<0.04$), MDS ($p<0.009$), and 5Gy+MDS ($p<0.04$).

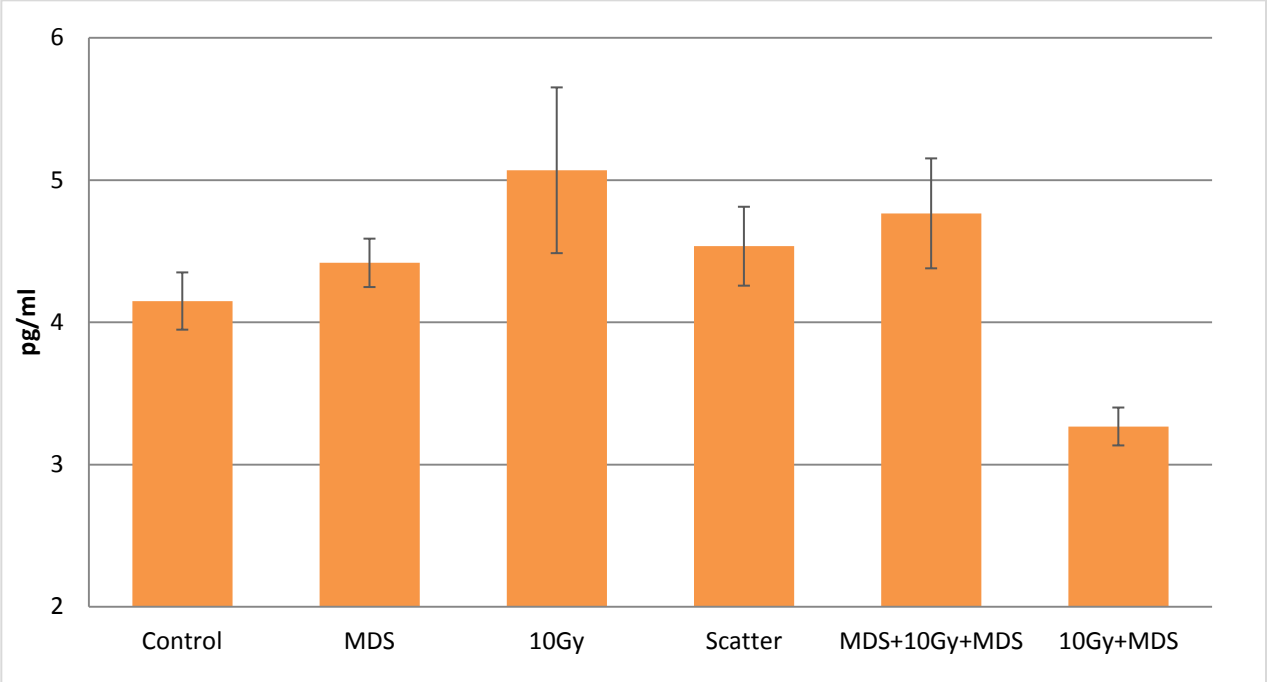

Figure 17: Effects of various treatments on circulating levels of IFN-γ in pg/ml at 30 days in phase two mice. Duncan's multiple range test indicated a difference between 10Gy and Control ($p<0.01$). There was also a significant difference between 10Gy+MDS and all other groups, Control ($p<0.007$), MDS ($p<0.001$), 10Gy ($p<0.00003$), Scatter ($p<0.0005$), and MDS+10Gy+MDS ($p<0.00009$).

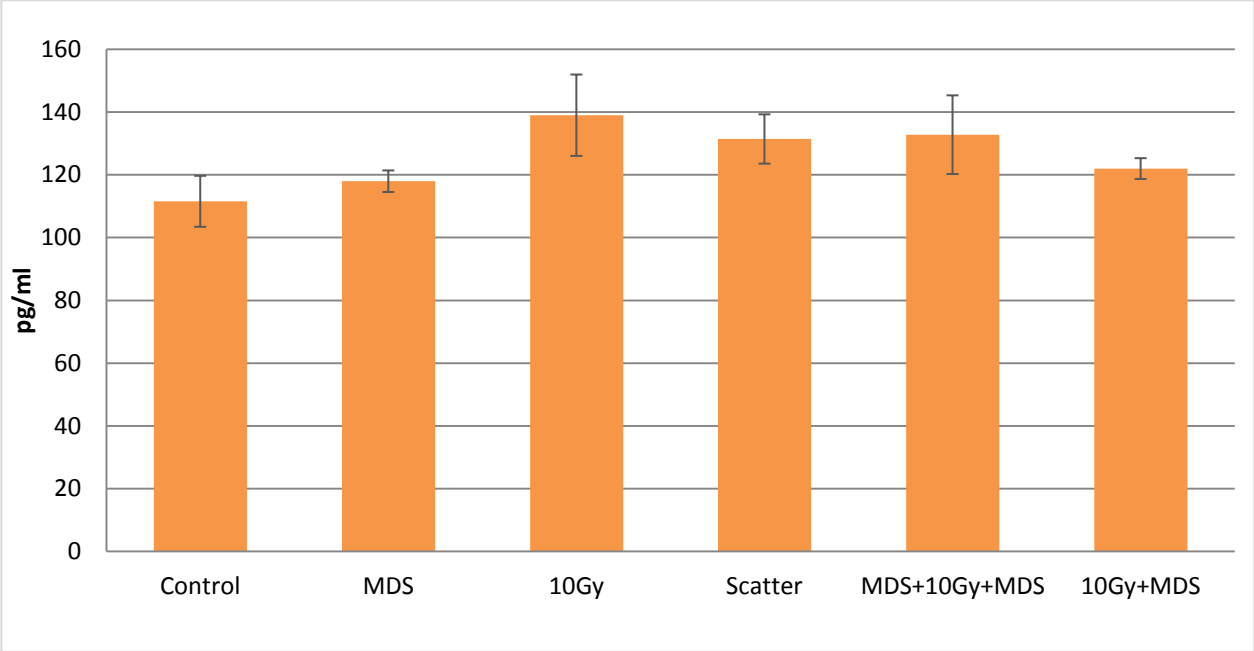

Figure 18: Effects of various treatments on circulating levels of IL-1β in pg/ml at 30 days in phase two mice. Duncan's multiple range test indicated a difference between Control and 10Gy ($p<0.008$), Scatter ($p<0.05$), MDS+10Gy+MDS ($p<0.04$). There was also a significant difference between MDS and 10Gy ($p<0.04$).

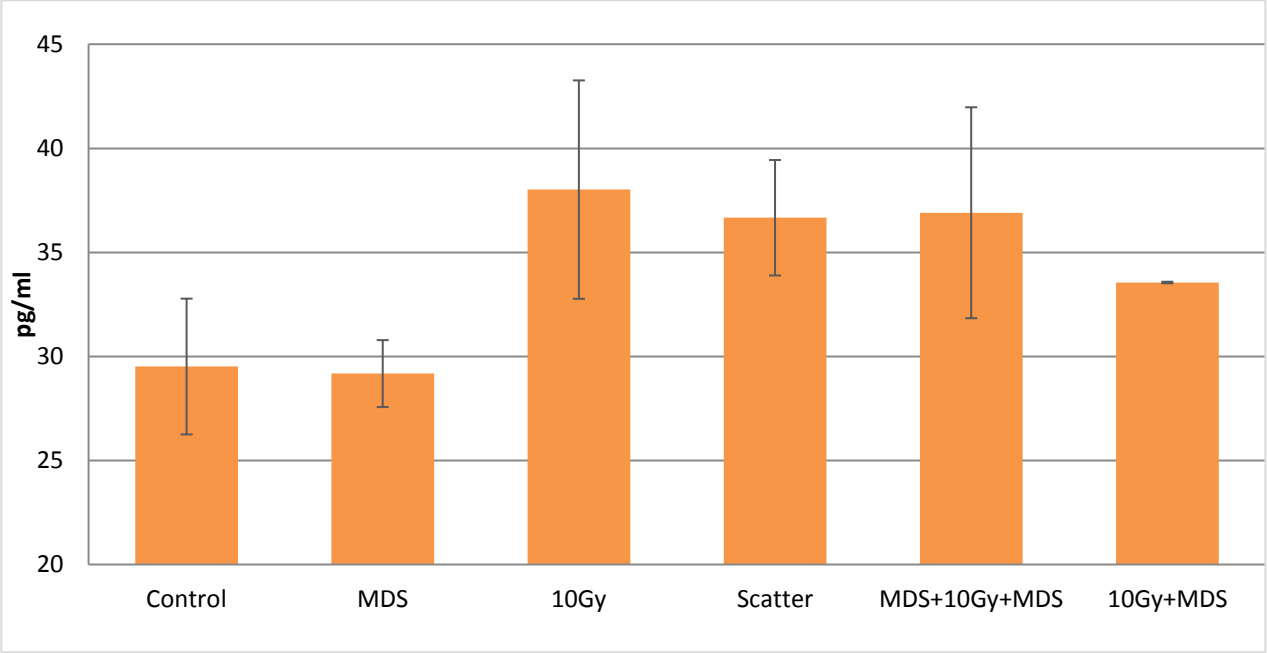

Figure 19: Effects of various treatments on circulating levels of IL-6 in pg/ml at 30 days in phase two mice. Duncan's multiple range test indicated a difference between 10Gy and Control ($p<0.04$) and MDS ($p<0.04$).

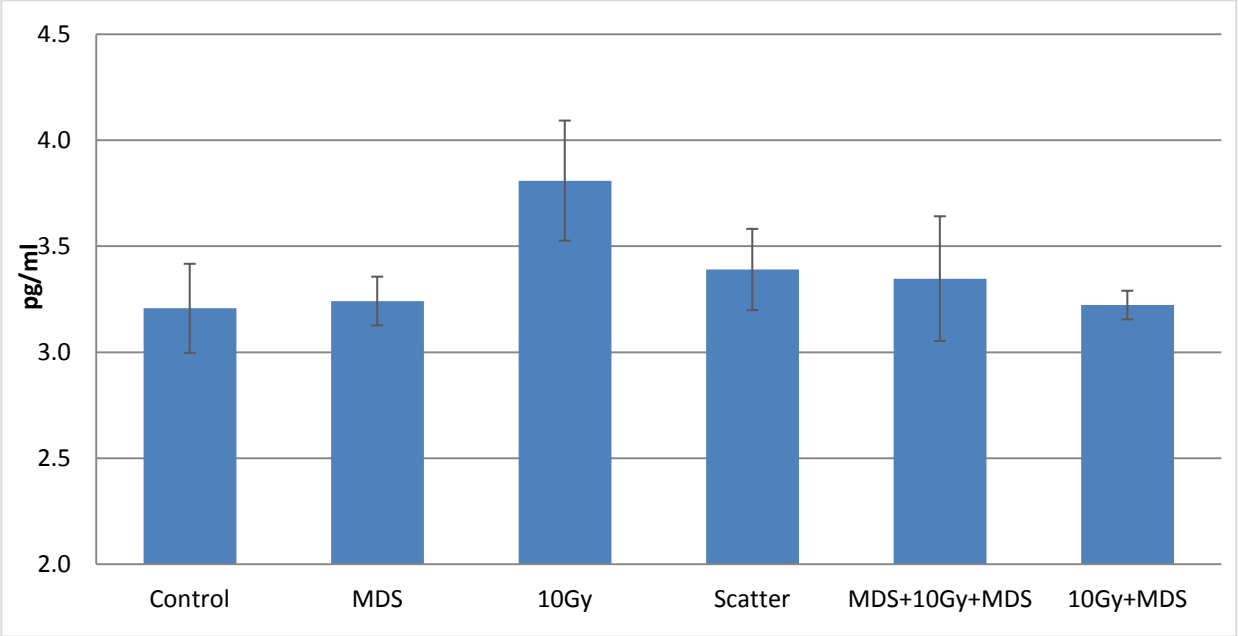

Figure 20: Effects of various treatments on circulating levels of IL-2 in pg/ml at 30 days in phase two mice. Duncan's multiple range test indicated a difference between 10Gy and Control ($p<0.04$), MDS ($p<0.04$), and 10Gy+MDS ($p<0.04$).

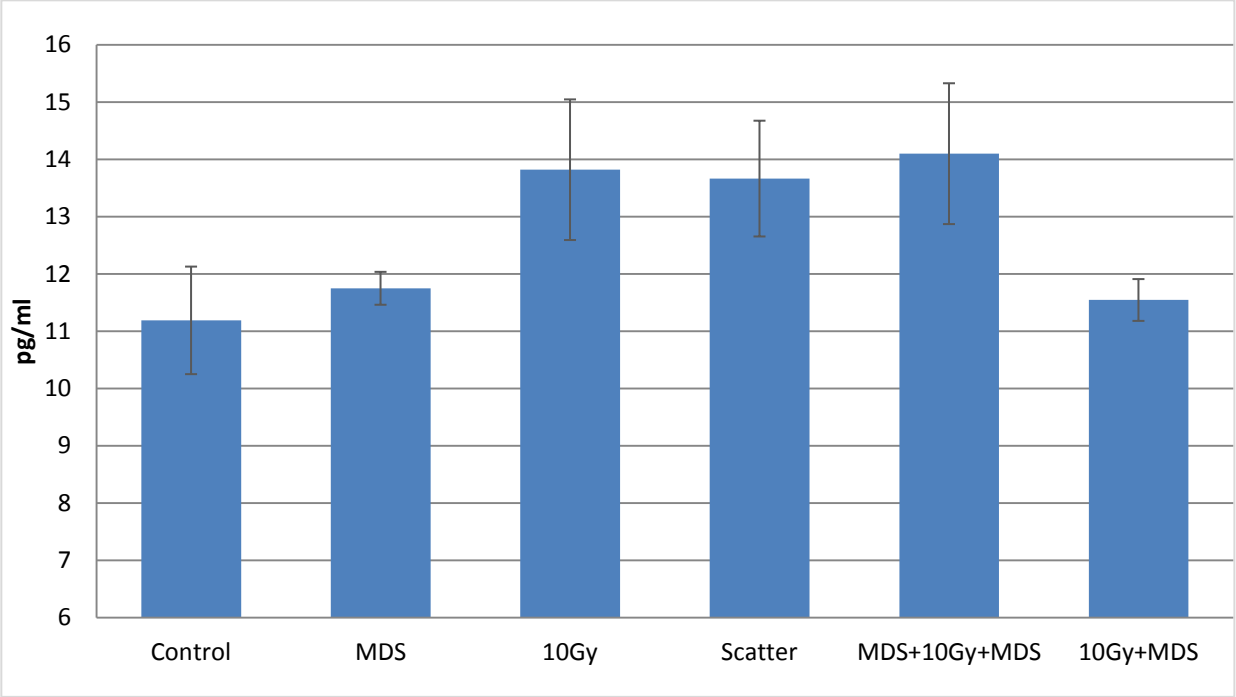

Figure 21: Effects of various treatments on circulating levels of IL-10 in pg/ml at 30 days in phase two mice. Duncan's multiple range test indicated a difference between Control and 10Gy ($p<0.05$), Scatter ($p<0.05$) and MDS+10Gy+MDS ($p<0.03$). There was also a significant difference between MDS+10Gy+MDS and 10Gy+MDS ($p<0.05$).

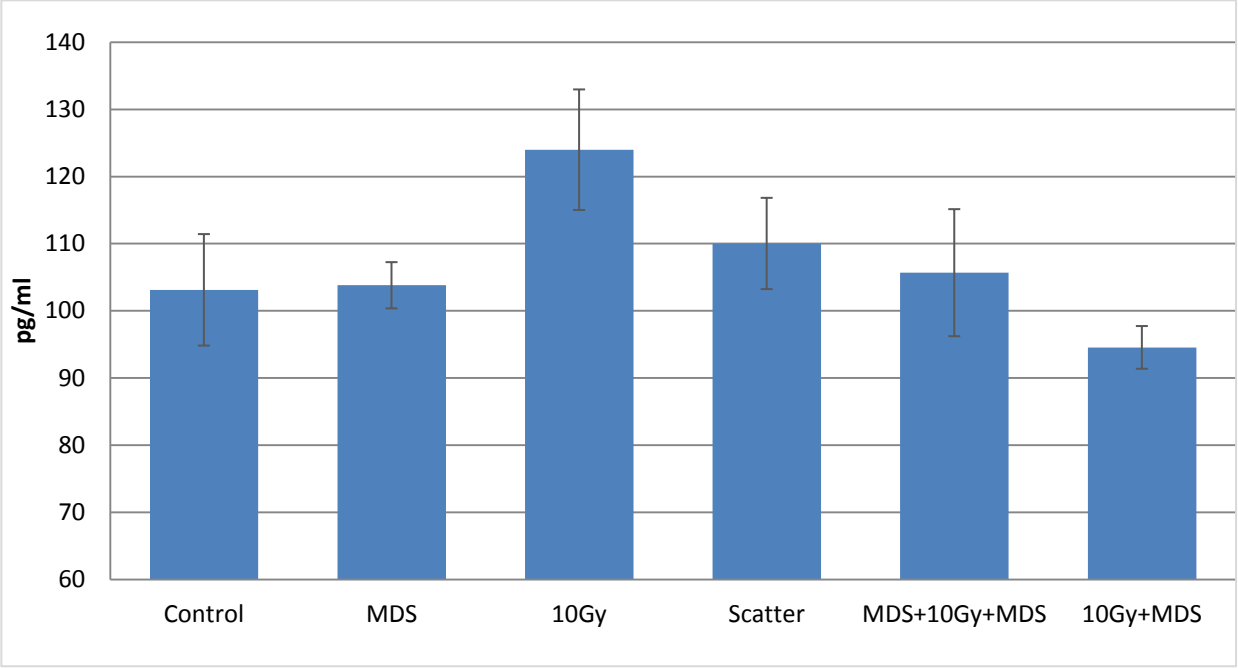

Figure 22: Effects of various treatments on circulating levels of IL-4 in pg/ml at 30 days in phase two mice. Duncan's multiple range test indicated a difference between 10Gy and 10Gy+MDS ($p<0.008$).

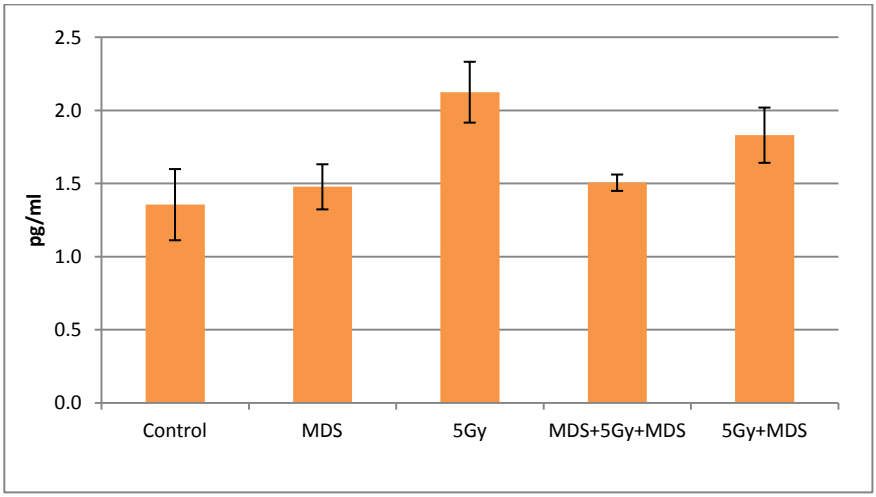

Figure 23: Effects of various treatments on circulating levels of TNF-α in pg/ml at 120 days. Duncan's multiple range test indicated a difference between 5Gy and Control ($p<0.02$), MDS ($p<0.04$), and MDS+5Gy+MDS ($p<0.04$).

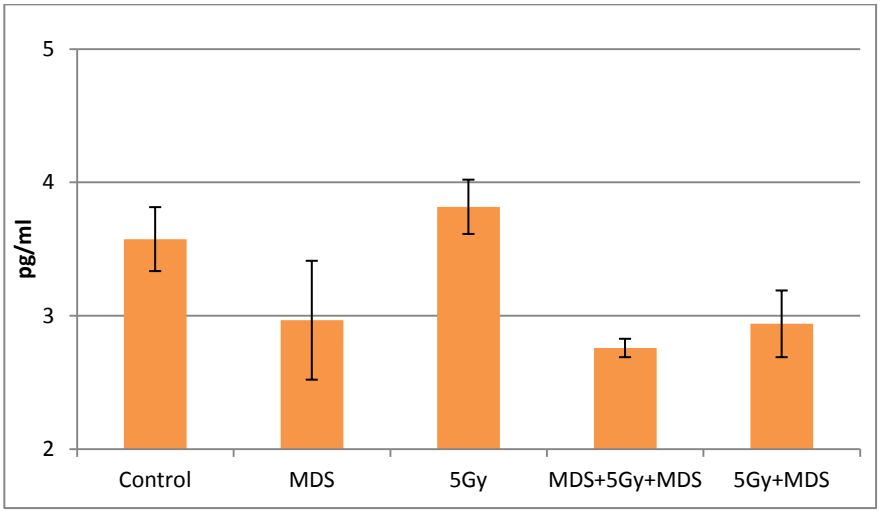

Figure 24: Effects of various treatments on circulating levels of IFN-γ in pg/ml at 120 days. Duncan's multiple range test indicated a difference between 5Gy and MDS ($p<0.05$), MDS+5Gy+MDS ($p<0.03$) and 5Gy+MDS ($p<0.05$).

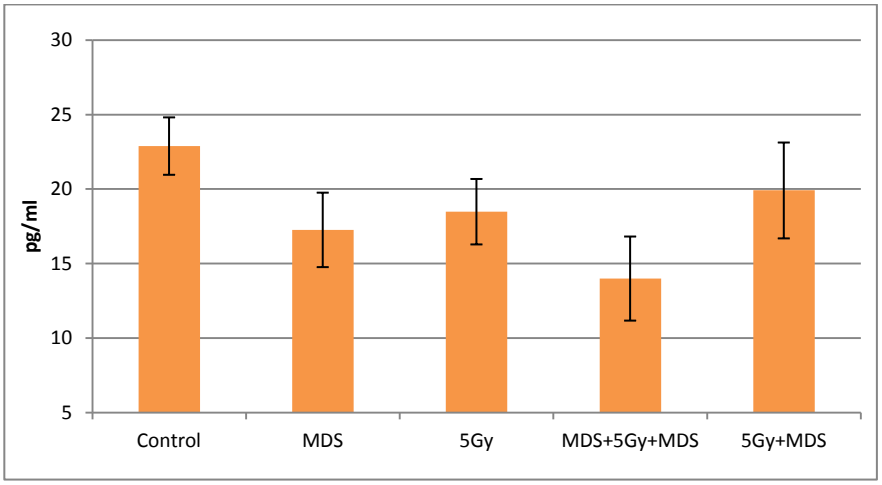

Figure 25: Effects of various treatments on circulating levels of IL-6 in pg/ml at 120 days. Duncan's multiple range test indicated a difference between MDS+5Gy+MDS and Control ($p<0.04$),

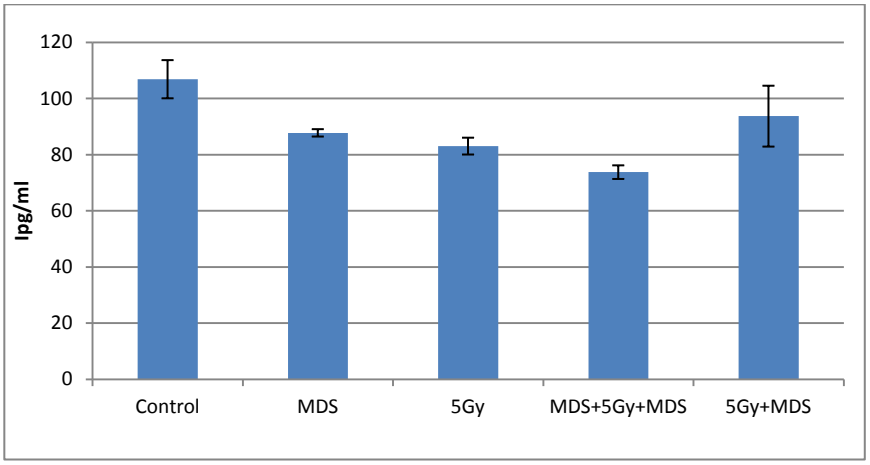

Figure 26: Effects of various treatments on circulating levels of IL-4 in pg/ml at 120 days. Duncan's multiple range test indicated a difference between Control and MDS ($p<0.05$), 5Gy ($p<0.02$) and MDS+5Gy+MDS ($p<0.002$). MDS+5Gy+MDS is also different from 5Gy+MDS ($p<0.05$).

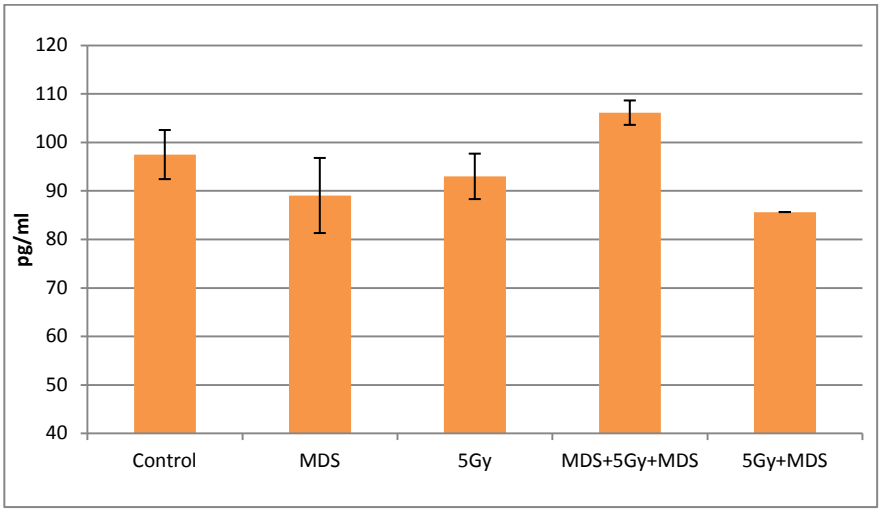

Figure 27: Effects of various treatments on circulating levels of IL-1β in pg/ml at 120 days. Duncan's multiple range test indicated a difference between MDS+5Gy+MDS and MDS ($p<0.03$), and 5Gy+MDS ($p<0.02$).

1.5 Summary and Discussion

Cytokine production.

Macrophages, by in large, are the primary producers of pro-inflammatory cytokines but many other cells can produce cytokines or upregulate cytokine production through signalling (Zhang & An, 2007). IL-1β, for example, is produced by many cells including monocytes, macrophages, fibroblasts, and endothelial cells (Copray et al., 2001). Almost all of the circulating immune cells can produce IL-10 including, macrophages, T-cells, B-cells, monocytes, and mast cells (Asadullah, Sterry, & Volk, 2003).

Helper T cells are a central secondary producer of most cytokines. Macrophages produce TNF-α, IFN-γ, IL-1, IL-6, and IL-10. T-cells produce TNF-α, IFN-γ, IL-1, IL-2, IL-4, IL-6 and IL-10. Neutrophils produce TNF-α. B-cells produce TNF-α and IL-10. Mast cells produce IL-4 (Zhang & An, 2007). IL-12p70 is produced primary by myeloid cells while controlled by helper T-cells. Monocytes can also produce IL-12p70 independently (Bekeredjian-Ding et al., 2006).

Macrophage activity is modified by exposure to radiation. Exposure to gamma rays has been shown to act as a priming influence, via incitation of IFN-γ, for sensitizing cells for cytotoxicity. This sensitization last for at least 24 hours and therefore would likely be present at the 2 day time point. Increased levels of TNF-α production by the macrophage, induced by outside influence or internal signalling, activates the macrophage for intracellular pathogen destruction and removal of apoptotic cells. Activated

macrophages show changes in 25% of observed genes but the impact of these changes are not yet well understood (Lambert & Paulnock, 1987; Mosser, 2003).

Cytokine Expression at 2 days.

Pro-inflammatory cytokines TNF-α, and IL-6 had expression lowered by MDS alone. MDS appears to have provided a protective effect when fed prior to irradiation. Being fed MDS post-irradiation failed to mitigate short term inflammation responses by these two cytokines. IL-12p70 had low expression in the MDS group. This supports the idea that MDS may suppress pro-inflammatory cytokine production. MDS failed to provide a beneficial effect on short term IL-12p70 levels when fed either prior to or post irradiation. IL-1β was high in the 5Gy+MDS group. This supports the implication, of TNF-α, and IL-6 patterns, that MDS fed post irradiation failed to mitigate short term inflammation.

IFN-γ had low expression in both the 5Gy and MDS+5Gy+MDS groups. This is generally considered a pro-inflammatory IL but MDS fed prior to irradiation seems to have produced the same effects as radiation alone.

Anti-inflammatory cytokines IL-2 and IL-4 showed low levels of expression in both 5Gy and MDS+5Gy+MDS groups. Levels of expression were high in the 5Gy+MDS group indicating that MDS fed after irradiation may help mitigate inflammation in the short term while pre-treatment with MDS was in fact harmful to short term inflammation control. Anti-inflammatory cytokine IL-10 had very high levels of expression in group 5Gy+MDS. This reinforces the IL-2 and IL-4 results indicating that MDS fed after irradiation may help mitigate inflammation.

Since inflammation on a systemic level is indicated by relative levels of pro and anti-inflammatory cytokines, the low levels of pro-inflammatory cytokine expression in MDS pre-fed groups fails to show the whole picture. Levels of cytokine expression are somewhat low across the board for group MDS+5Gy+MDS and inflammation effects may be more serious than indicated when looking at isolated cytokines. Further indicators of stress such as fibrosis or behaviour changes should be examined to give a more complete picture (See Ch. 2 for behavioural data). Levels of 8-OHdG (Ch. 4) provide a clearer picture of possible causes for inflammatory cytokine signalling expression.

Diet compliance was not ideal at this time point in the 5Gy+MDS group. This partial compliance with the feeding regime may have decreased applicable results. Already promising preliminary findings should prompt further investigation with strategies to increase diet compliance.

Cytokine Expression at 30 days (Phase One).

At 30 days cytokine expression in the 5Gy group and 5Gy+MDS groups both took dramatic downturns for many of the cytokines. Expression in group 5Gy was significantly low for all cytokines excluding IL-12p70. Pro-inflammatory cytokines TNF-α, IFN- γ, IL-1β, and IL-6 all displayed a similar pattern. Expression levels in Control, MDS, and MDS+5Gy+MDS were higher than in group 5Gy and for IFN-γ, IL-1β and IL-6. Control, MDS and MDS+5Gy+MDS also had higher expression levels than 5Gy+MDS for IFN-γ, IL-1β and IL-6.

Anti-inflammatory cytokines IL-2, IL-4 and IL-10 had low expression in 5Gy similar to the pro-inflammatory cytokines. Both IL-2 and IL-4 group 5Gy+MDS

expression levels were statistically lower than MDS+5Gy+MDS levels in addition to control and MDS levels indicating that feeding post-irradiation failed to rescue anti-inflammatory cytokine expression as well as feeding pre-irradiation. IL-10 had expression levels in group 5Gy+MDS lower than MDS. MDS+5Gy+MDS levels were not different from any other groups. The cytokine's expression supports the pattern of failure to rescue as capably as being fed MDS prior to irradiation. Cytokine IL-12p70 was again the only cytokine that did not fit the pattern of expression. IL-12p70 expression levels were highest in the 5Gy group differing from the control, MDS and 5Gy+MDS.

Similarly to the 2 day time point, a more accurate measure of systemic inflammation is the balance between pro-inflammatory and anti-inflammatory cytokines. Low levels of expression in the irradiated group could point towards cell killing or blocking of key cell types necessary to elevate cytokine production. Macrophages are usually stimulated at doses of 10Gy or less but the pattern is irregular (S.-Z. Liu, 2003). Macrophages exposed to cytokines like IL-4 block cytotoxic lymphocytes and promote remodeling of tissue (Ruffell, DeNardo, Affara, & Coussens, 2010).

Diet compliance in the group fed post irradiation had recovered but had not been steady for more than two weeks prior to testing (unpublished data). Overall inflammation in the post-fed MDS group appears to be low even with less than ideal MDS compliance. Two weeks may have been too little time to build up effective levels of active ingredients in the body sufficient to counter the damage that had already propagated over the previous weeks and even greater results may be possible with better diet compliance.

Cytokine Expression at 30 days (Phase Two).

Cytokine expression in the 10Gy group was high for all pro-inflammatory cytokines. IL-1β showed high levels in all groups that had received radiation except 10Gy+MDS. MDS fed post-irradiation was able to rescue cytokine expression to control-like levels. This is reinforced by results of IFN-γ which had significantly lowest cytokine expression in the 10Gy+MDS group. IL-6 showed high impact of 10Gy radiation compared to control and MDS levels but did not show significant protective impact of MDS fed post irradiation.

Results seem to get more interesting when one examines the anti-inflammatory cytokines tested. Positive effects of MDS fed post-irradiation are not clear. MDS fed post-irradiation did not appear to increase anti-inflammatory cytokine production. It could be possible that since pro-inflammatory cytokines were also very low that systemic inflammation levels had already been dealt with or that systemic inflammation never occurred to the level that it did in the irradiated only group or even in the group fed MDS both before and after irradiation. Il-2 displayed high expression levels in the 10Gy group.

IL-10 expression was high in the 10Gy, Scatter and MDS+10Gy+MDS group compared to the control. IL-4 was expressed more in the 10Gy group then the 10Gy+MDS group. MDS fed prior to irradiation showed little to no positive benefit in this trial with elevated or near control level of pro-inflammatory cytokines and near-control levels of anti-inflammatory cytokines.

Diet compliance in all groups in phase two was excellent. This is of particular interest for groups fed post-irradiation. Since results of this very clinically relevant group

continue to show promise with regards to decreased pro-inflammatory reaction further study of effective administration timelines is warranted. Initial feeding of the bagel was delayed by several hours in order to decrease association with the irradiation procedure. Long term compliance was better achieved this way but crucial hours of impact may have been missed. This MDS may indeed mitigate much of the chronic inflammation begun by the initial stress event but only if administered quickly. Small differences in administration timelines may make significant differences and this should not be ignored. Additional time points such as were examined in phase one mice, may give a better picture as well.

Cytokine Expression at 120 days.

A pattern of low expression is evident in the group fed MDS both before and after irradiation in several of the pro-inflammatory cytokines. TNF-α and IFN-γ expression is lower in groups fed MDS continuously than in the irradiation only group. IL-6 expression is lower when continuously fed MDS than in the control group. Pro-inflammatory cytokine IL-1β breaks the trend with high expression in the group continuously fed MDS; higher than both the control and the post-irradiation MDS fed group.

The only anti-inflammatory cytokine to show significance, IL-4, had low levels of expression in the continuously fed group compared to the control. Cytokines IL-12p70, IL-2, and IL-10 did not display any significant differences between groups. This makes it more difficult to draw conclusions at the 120 day time point since systemic inflammation is really a product of the balance of cytokines.

Conclusion

2 days.

MDS alone provided decrease in pro-inflammatory cytokines. IFN-γ, IL-2 and IL-4 show failure of continuous MDS to protect but IL-6 and TNF-α expression was decreased by continuous MDS. MDS post-irradiation failed to decrease pro-inflammatory cytokines but increased anti-inflammatory IL-2, IL-4, and IL-10. Pro-inflammatory cytokine production was suppressed by MDS in both MDS alone and continuous MDS groups except for IL-12p70. IL-12p70 is produced primary by myeloid cells, in contrast to many of the other pro-inflammatory cytokines produced mainly by macrophages (Bekeredjian-Ding et al., 2006).

30 days.

MDS alone provided an overall increase in cytokine expression. 5Gy resulted in overall suppression of cytokines (not IL-12p70). Feeding MDS continuously decreased cytokine profiles to control levels. Anti-inflammatory cytokine levels in the MDS continually fed group were rescued better than post-feeding at this time point but this may have to do with overall decreased inflammatory state of the post-fed MDS group (See Ch. 4).

MDS post-irradiation, at this time point, decreased expression of pro-inflammatory IFN-γ, IL-1β, and IL-6, but failed to increase expression of anti-inflammatory cytokines to levels of continuously MDS fed mice meaning that cytokine levels were low across the board. Il-6 expression in particular is very low compared to

other groups indicating a decreased overall inflammation status. Overall expression is balanced.

120 days.

Continuous feeding of MDS has lowered pro-inflammatory status as indicated by TNF-α, IFN-γ, and IL-6. Persistence of anti-inflammatory inhibition in group continuously fed MDS receives some support from levels of IL-4 but levels of IL-2 and IL-10 are unaffected. Since pro-inflammatory levels have subsided this may be more indicative of overall decreased inflammatory status.

Phase One Mice.

In conclusion, in phase one mice at all time points, pre-feeding MDS seems to protect against strong acute pro-inflammatory reaction but also blocks rapid upregulation of anti-inflammatory cytokines. Preliminary data indicates that the levels of 8-OHdG may be slightly decreased in this group compared to the irradiated and control groups. Larger studies are necessary to indicate if this result is significant. Feeding after irradiation allowed cytokine levels to be quickly upregulated after stress but cytokine levels dropped significantly after the 30 day time point. Since acute responses of pro-inflammatory cytokines prompt tissue repair this may be the best scenario. Perhaps the MDS acts to boost the immune system after stress and by the 30 and 120 day time points damage is healed and inflammation is no longer necessary. Continuous MDS feeding would have protected against some of the damage and require less of a response at 2 days supported by evidence of inflammation levels near control levels after 30 and 120 days.

Phase two mice.

The irradiated group exhibited increased pro-inflammatory markers IFN-γ, IL-1β, and IL-6. Continuous MDS feeding expressed pro-inflammatory IFN-γ at a higher level than post-irradiation feeding. Continuous MDS feeding also expressed increased levels of anti-inflammatory cytokine IL-10 when compared to the MDS post-irradiation group. MDS post-irradiation was able to decrease pro-inflammatory cytokine expression IL-1β, and IFN-γ but IL-6 levels were close to control. Levels of anti-inflammatory cytokines were also low, indicating that overall cytokine profile was normalized. An earlier time point is necessary to observe if the pattern of dramatic upregulation of cytokine response and then drop in cytokine levels, seen at lower radiation doses, holds true at higher doses.

Cytokine Balance.

Excessive pro-inflammatory cytokine expression is connected to multiple pathologies including diabetes, cardiovascular disease, and cancer. Prevalence of excessive anti-inflammatory cytokines is connected with immune suppression and infection risk (Barrett, Dai, Gamberg, Gallant, & Grant, 2007). Good health depends on a proper balance of pro and anti-inflammatory cytokines. Two mechanisms may have been at play influencing the cytokine response. Firstly, ingredients of MDS may have blocked damage and prevented the necessity of a dramatic cytokine response. This is supported by the quick return of continuously fed MDS group cytokine expression returning to normal after an onslaught of stress caused by radiation. Secondly, ingredients of MDS may be down regulating defense systems and stress pathways. This may slow down response times as the functional ability of the body to protect and heal from stressors could have atrophied. Down regulation of macrophages seems a likely culprit since most cytokines

are produced by macrophages but IL-12p70 is produces primarily by myeloid cells. IL-12p70 levels responded differently to MDS feeding schedules than most of the other cytokines.

Being fed MDS only after irradiation would avoid the issue of immune system atrophy. This seems to be supported by high expression of pro-inflammatory cytokines but quick expression decline as the upregulated system heals damage. Perhaps the MDS would be most effective if cycled in order to avoid some effects of immune system downregulation.

CHAPTER 2:

MDS AND IMPACTS ON RECOGNITON OF NOVELTY

2.1 Introduction

Memory is an integral part of daily life. From remembering to put on pants to remembering that you left the oven turned on, without the ability to recall past behaviours and events, quality of life is severely compromised. One of the central features of chemofog is disruption of memory recall. Memory effects in humans can be easily accessed through written or verbal testing and patients can self-advocate for a more complete understanding of radiotherapy or chemotherapy side effects. Subtle changes in cognitive ability can produce frustration and dramatic shifts in patient's self-perception but can be difficult to quantify. Sophisticated computer testing is better able to find changes but confounding factors continue to make assessment difficult in patients (Servaes, Verhagen, & Bleijenberg, 2002). Animal testing allows for the elimination of many confounding factors but behavioral effects are still difficult to quantify. Memory testing in animals can be a difficult endeavour due to difficulty designing tests that rely on memory but not other factors such as motivation and without external pressure (Antunes & Biala, 2012).

Novelty testing – Test sensitivities.

These procedures allows for the observation of a natural preference of mice toward novel objects without positive or negative reinforcement. Memory consolidation is considered hippocampus-dependent. When a situation includes novel aspects, the

memory must be recalled in the labile phase and organized in a process called reconsolidation. The dorsal hippocampus is the key brain region involved when spatial or contextual information is a factor (Goulart et al., 2010) such as in the Novel Placement Recognition (NPR) test. Neurogenesis in the hippocampal region has been strongly connected to spatial memory consolidation. The perirhinal cortex is highly integrated with the hippocampus but is responsible for short-term object recognition necessary for the Novel Object Recognition (NOR) test (Reger, Hovda, & Giza, 2009). Perirhinal function is linked to recognition of the object itself, while the hippocampus is involved in encoding and recalling the experience of the object (Hammond, Tull, & Stackman, 2004). The hippocampus is not key to the storage of information since in some contexts novel object recognition can in certain circumstances take place during a time when the hippocampus is incapacitated (Broadbent, Gaskin, Squire, & Clark, 2010) but is fundamental in novelty recognition since it is used to compare old memories with new (Clarke, Cammarota, Gruart, Izquierdo, & Delgado-García, 2010). Novel object testing provides a measure of working memory and executive function in mice and is considered sensitive to hippocampus neurogenesis (Sarkisyan & Hedlund, 2009), perirhinal cortex function, and function of the dentate gyrus (Goulart et al., 2010).

Failure to Discriminate.

Novelty produces two conflicting drives when presented to a mouse. There is a novelty exploration drive, a tendency to explore new objects. There is also an anxiety based avoidance behaviour observed (Powell, Geyer, Gallagher, & Paulus, 2004). A likely driver of novelty seeking is foraging behaviour. Attention to novelty ensures that

new threats or benefits of a changing environment are explored. The rewards inherent in novelty seeking behaviour share a common pathway with addictions such as morphine and mice that display addicted behaviour are predisposed to novelty seeking. Since novelty is not rewarding in and of itself, some have proposed that this internal dopamine driven reward similar to addiction satisfaction is an important driver in mouse novelty seeking (Powell et al., 2004; Zheng et al., 2003).

Neophobia is considered an indicator of anxiety and therefore novel object discrimination is strongly impacted by stress. Lack of discrimination may also be attributed to a change in perception of danger. If the animals are sufficiently stressed by the change in environment, or perhaps by the way they were handled, it may stress the animal sufficiently such that they reassess both objects. A stressed animal may reassess each object based on threat level, animation, and scent rather than on familiarity (Ennaceur, Michalikova, & Chazot, 2009).

2.2 Methods

Animals.

Two strains of male mice were kept, C57Bl/6J and Tg (Thy1-EGFP) MJrs/J mice (Thy1-GFP). Thy1-GFP mice are on a background of C57 mice and have a green fluorescent protein expressed in specific brain regions. All care was provided as outlined in the Canada Council on Animal Care guidelines. Mice were fed and watered *ad libitum* and housed individually. They were exposed to a 12hr/12hr day/night photoperiod.

Groups.

Mice were assigned at random to trial groups upon arrival. Seventy phase one mice were split into 5 groups. Forty phase two mice were split into 6 groups.

Multiple Ingredient Dietary Supplementation.

A multiple ingredient supplement was originally designed to ameliorate the deterioration of five mechanisms of aging(Aksenov, Boreham, & Rollo, 2014; Lemon, Rollo, & Boreham, 2008; Lemon, Rollo, McFarlane, & Boreham, 2008). Original daily doses were determined as based on recommended doses for adult humans and adjusted for the higher metabolic rate of mice and the adjusted for body size. The MDS was put into solution and was soaked into pieces of bagel. Daily bagel consumption was recorded and after an adjustment period was eaten voraciously.

Novel object recognition.

Mice were habituated to an empty Plexiglas arena (40*40*30cm L×W×H) for 10 minutes per day for 3 consecutive days. Low level lighting was used in order to provide a calming environment. Testing took place on the fourth day. The arena was prepped with 2 objects of the same shape and colour. After 5 minutes of familiarization the mouse was removed and placed in his home cage for 5 minutes. The arena and objects were cleaned with Quatricide to remove scent trails. One familiar object was replaced in the arena while a novel object was added in the position of the second object (Fig. 28). One minute of free exploration was video recorded and later the interaction time with each object was recorded according to an adaptation of the procedure outlined by Ennaceur (2010) and

Gaskin et al. (2010). Each mouse was tested only once with both the NOR and NPR tests. A ratio was calculated as a measure of preference for novel object:

$$\text{Preference for novel object ratio} = \frac{\text{Total time exploring novel object}}{\text{Total time exploring familiar object}}$$

Novel Placement Recognition.

Mice were habituated to an empty Plexiglas arena (40*40*30cm W×L×H) for 10 minutes per day for 3 consecutive days the week following NOR testing. Low level lighting was maintained. Testing took place on the fourth day and the arena was prepped with 4 objects of the different shapes and colours. After 10 minutes of familiarization the mouse was removed and placed in his home cage for 5 minutes. The arena and objects were cleaned with Quatricide. One familiar object was replaced in the arena while a novel object was added in the position of the second object (Fig. 29). One minute of free exploration was video recorded and later the interaction time with each object was recorded according to an adaptation of the procedure outlined by Hale and Good (2005). A ratio was calculated as a measure of preference for novel object placement:

$$\text{Preference for novel object placement} = \frac{\text{Total time exploring novel object placement}}{\text{Total time exploring familiar object placement}}$$

Statistical analyses.

The effects of MDS treatment on both novel and familiar object exploration was examined using ANOVA and post-hoc Duncan's tests between all treatment groups. Effects of time point were non-significant and data between time points was pooled. Differences between mouse strains in NOR were also non-significant and were pooled for phase two mice. Mice strains data was non-significant and pooled for NPR testing. Group

size was Group size ranged from n=5 (10Gy+MDS) to n=10 (pooled control and pooled MDS in phase two). Most groups contained n=5

2.3 Results

Novel object recognition.

In phase one the 5Gy+MDS group strongly preferred the novel object above the familiar object interacting 4 times more with the novel object. This was significantly more than the control ($p<0.009$) and MDS ($p<0.005$) groups (Fig. 30). In phase two the scatter group discriminated strongly towards the novel object, interacting 4 times more with the novel object than the familiar. This was significantly different than the control and MDS alone groups ($p<0.030$ and $p<0.012$, Fig. 31). The 10Gy+MDS group preferred the novel object significantly more than the MDS group ($p<0.05$) (Fig. 31).

Novel placement recognition.

Phase one of the experiment showed no significant differences between groups when Analysis of Variance was performed (Fig. 32). During phase 2 of the experiment, the 10Gy+MDS group interacted with the object placed in a novel location almost 4 times longer than the familiar object. This was a significantly greater novel object preference than groups MDS ($p<0.004$), 10Gy ($p<0.05$), and MDS+10Gy+MDS ($p<0.04$) (See Figure 33).

2.4 Figures

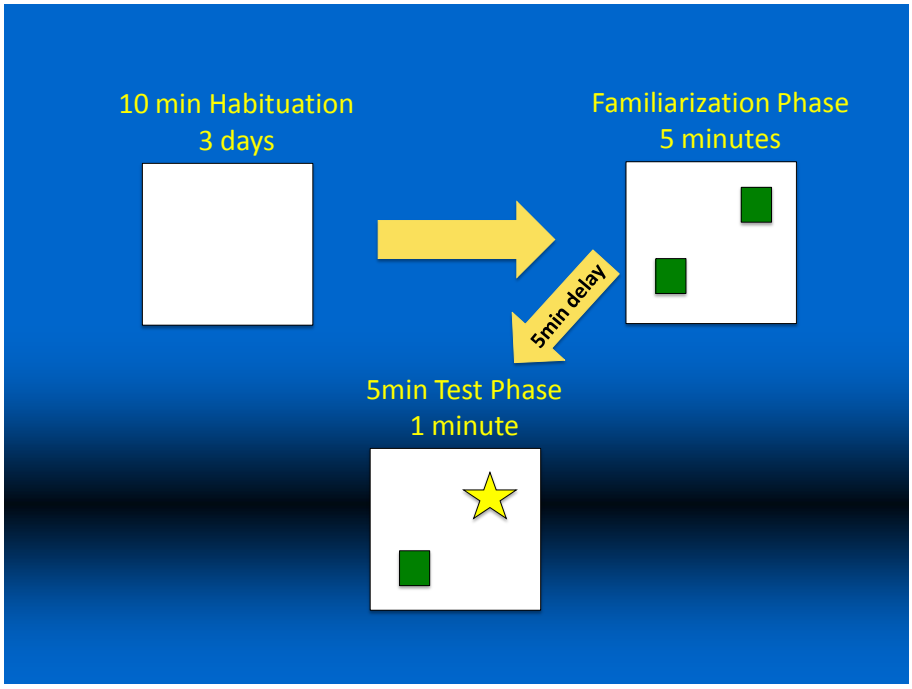

Figure 28: Hippocampus-dependent memory function: novel object recognition (NOR) task.

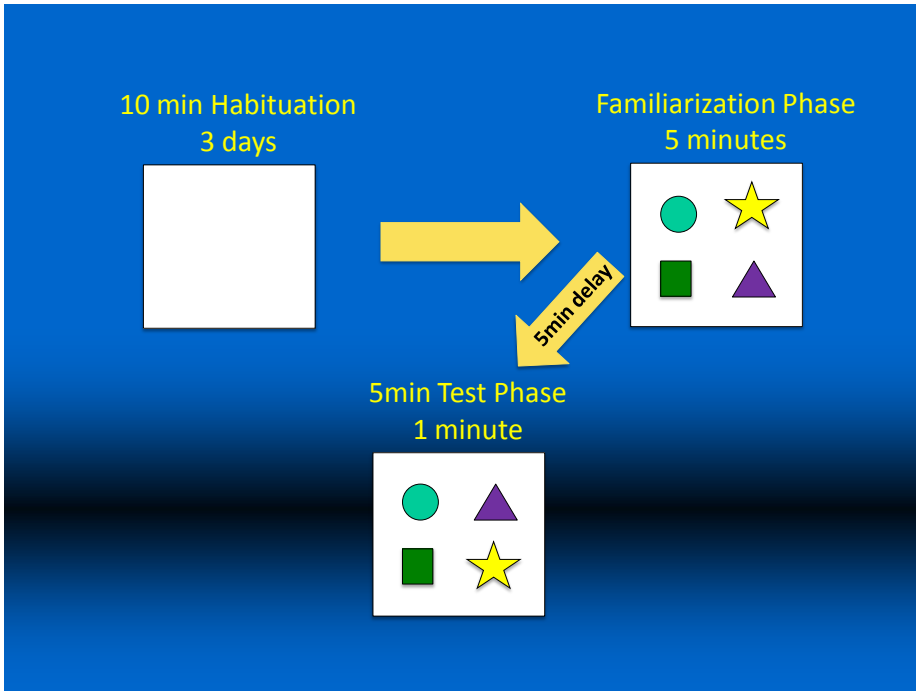

Figure 29**:** Hippocampus-dependent memory function: novel placement recognition (NPR) task.

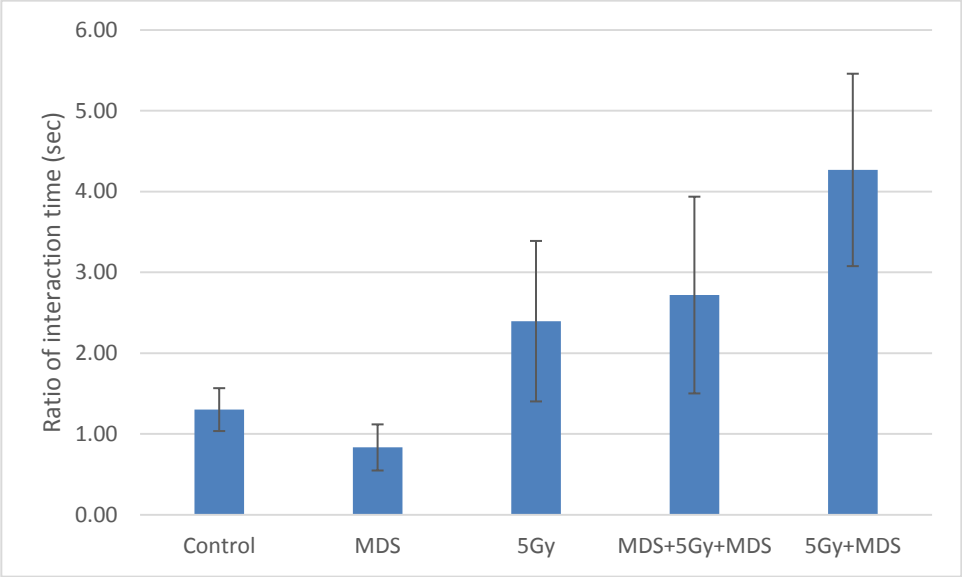

Figure 30: Novel Object recognition test preference for novel object. Ratio of interaction time with novel object by interactions time with familiar objects. The 5Gy+MDS group preferred the novel object significantly more than the control ($p<0.04$) and MDS ($p<0.02$) groups.

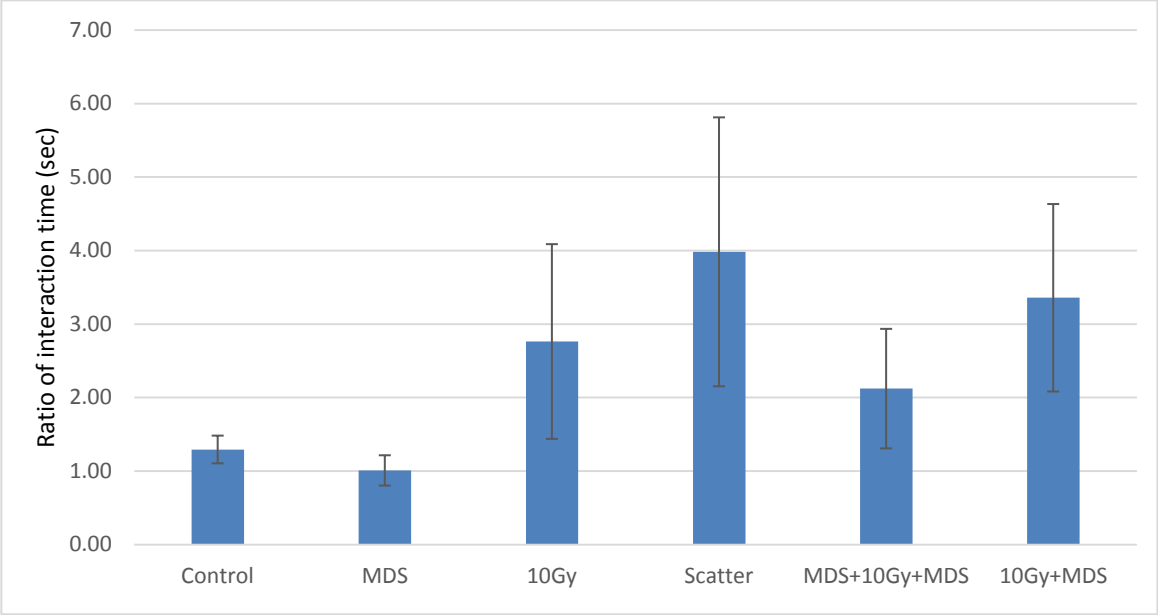

Figure 31: Novel Object recognition test preference for novel object. Ratio of interaction time with novel object by interactions time with familiar objects. The Scatter group preferred the novel object statistically more strongly than the control ($p<0.03$) and MDS ($p<0.02$) groups. The 10Gy+MDS group preferred the novel object significantly more than the MDS group ($p<0.05$).

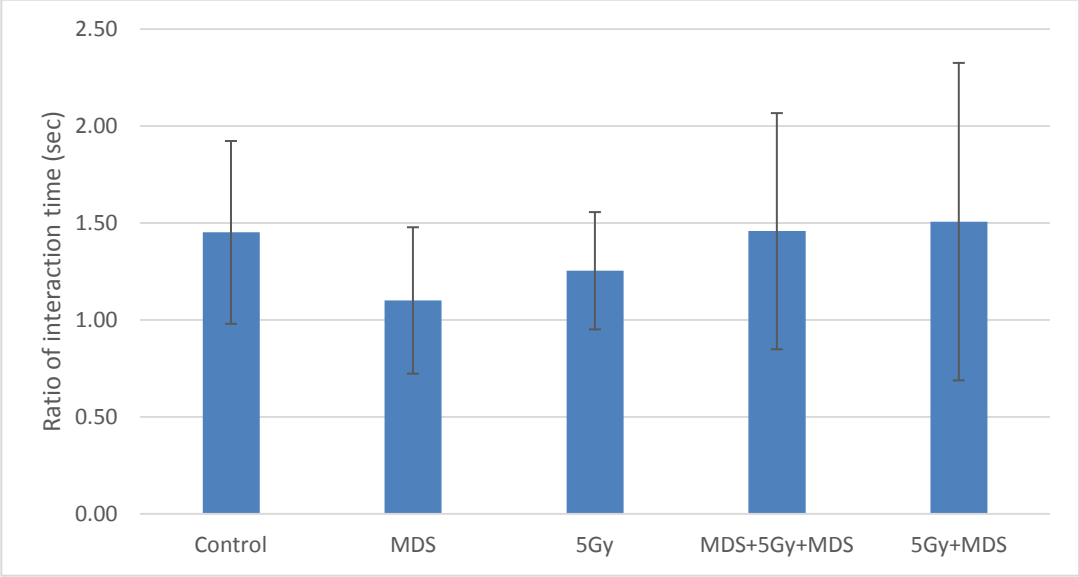

Figure 32: Novel placement recognition test preference for novel placement. Ratio of interaction time with novel objects/ interaction time with familiar objects. Analysis of variance indicated no significant differences between groups.

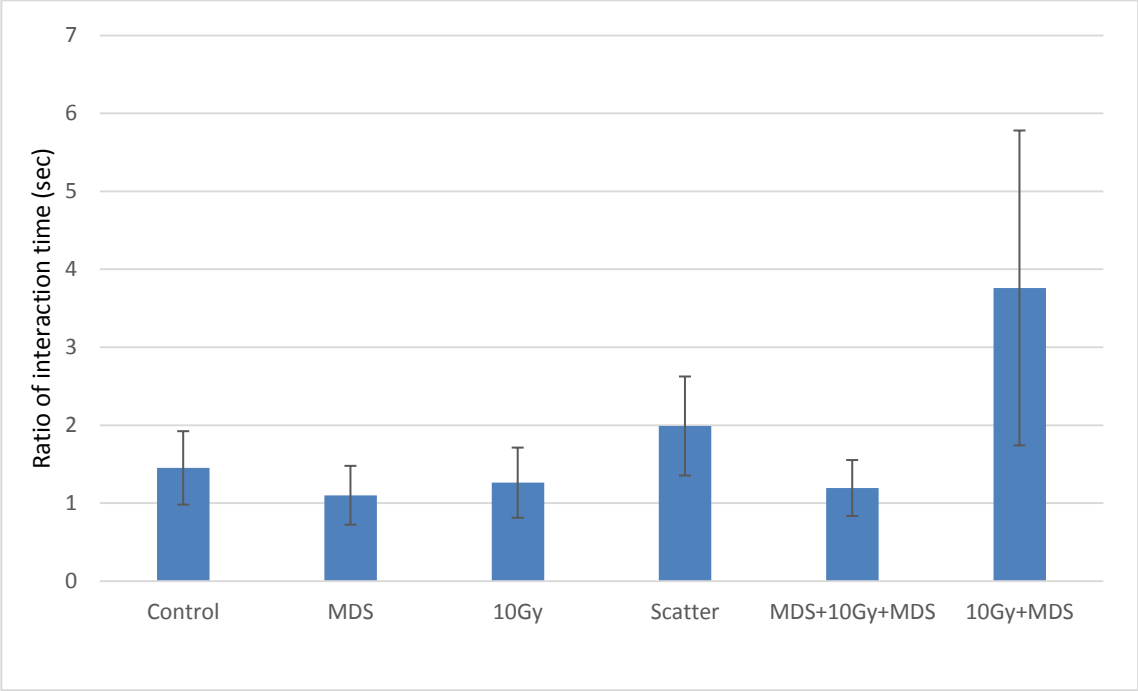

Figure 33: Novel placement recognition test preference for novel placement. Ratio of interaction time with novel objects/ interaction time with familiar objects. The 10Gy+MDS group displayed significantly greater novel object preference than groups MDS ($p<0.004$), 10Gy ($p<0.05$), and MDS+10Gy+MDS ($p<0.04$).

2.5 Summary and Discussion

Novel object recognition (NOR) testing is normally a very clean measure of memory since these procedures allow for the observation of a natural preference of mice toward novel objects without positive or negative reinforcement (Goulart et al., 2010). If mice do not prefer to explore the novel object that indicated a failure to recognize that the object is novel. Novelty of an object is the main impetus for exploration in mice. Novelty-seeking is however considered a measure of personality and therefore a wide range of novelty preference is expected (Dulawa, Grandy, Low, Paulus, & Geyer, 1999). Control mice spent 29% more time with the novel object (Fig. 30) during NOR testing. The control mice also spent 45% more time with objects in the novel location during NPR testing (Fig. 33). This discrimination indicated that the test parameters allowed adequate time for familiarization with the objects and later recall, recognition and novelty exploration.

MDS only fed mice did not appear to differentiate between novel and familiar objects in phase one or phase two or NPR. Statistically MDS fed mice were not different from the control. It is also possible that the time frame analyzed was too long and allowed for supplemented mice to become familiarized with both objects before the end of the observation period. If the mice were familiarized with both objects the inherent exploratory behaviour is lost and the mouse will not discriminate between objects. Inability or unwillingness to differential between novel and familiar objects is often interpreted as a sign of anxiety (Powell et al., 2004).

At 30 days the cytokine expression in the MDS group was quite elevated. Conclusions are difficult to draw but inflammation status as seen in Ch. 1 provides insight. Pro-inflammatory cytokines in phase one were elevated in the MDS group. IL-6 is especially seen as a marker for high inflammation status (T. Hayashi et al., 2012) and was highest in MDS only and MDS+5Gy+MDS groups in phase one. In phase two mice, mice fed MDS had decreased levels of IL-6 and increased levels of IL-10. In mice fed post-irradiation overall cytokine levels were low.

Both phase one and phase two mice in the MDS+10Gy+MDS group did not perform significantly better than the control. Some of the components of the MDS are shown to act hormetically (Calabrese et al., 2010; Mattson, 2008). Stress resistance pathways are activated and can act to reduce risk of disease. These stress resistance mechanisms are already activated and may then be overwhelmed by further stress impacts of radiation (Gundala & Aneja, 2014). This goes a long way in explaining the poorer than expected discrimination in mice continuously fed MDS before and after irradiation.

Mice which underwent radiation, and mice that were fed MDS continuously, as well as mice fed after irradiation exhibited increased exploratory behaviour towards the novel object resulting in a greater number of interactions and more time exploring. Of the phase one mice only the group fed MDS post-irradiation was statistically different from the control and MDS only group in phase one. Phase two mice displayed a similar pattern. The group exposed only to the control dose of scatter radiation was significantly more discriminating than the control or MDS mice. Phase two mice fed MDS post-irradiation also discriminated more strongly than the MDS only group. The advantageous

effect in the post-irradiation fed group indicated that MDS still has a benefit to confer. Since MDS is sourced from natural extracts and phytochemicals, influences on biomarkers of cognition may be nuanced (Deorukhkar et al., 2007).

A scatter radiation dose of 0.5Gy may be acting hormetically. The linear no threshold model of radiation assumes that all radiation doses are detrimental and that damage is linearly related to dose. However, lower dose radiation may not carry the same risks and many researchers believe there may be a threshold below which there is no increased risk and in fact low doses of radiation may be advantageous. Radiation workers are an ideal group to study low dose radiation since their doses are generally low but very accurately measured due to personal dosimeter safety regulations. The International Commission on Radiological Protection changed the recommended dose limit in 1930 to 0.5Gy per year. After this limit decrease mortality among radiation exposed physicians decreased to average levels (Yoshinaga, Mabuchi, Sigurdson, Doody, & Ron, 2004). British radiologist in particular experienced very low mortality rates, lower than the average of medical practitioners, after the recommended protection limit was decreased indicating that at levels equal to or below the recommended 0.5Gy dose radiation can be beneficial (Doll, Berrington, & Darby, 2005). Another study examined medical exposure to x-rays and found that excess cancers were found only above a dose of 0.5Gy (Tubiana, 2008).

Limitations.

There are several limitations that must be acknowledged. Phase one, C57, mice receiving MDS post irradiation had diet compliance decreased by radiation induced

nausea. Behaviour effects in this treatment are more clearly shown in the phase two, GFP, mice. Sample sizes of 5 to 10 mice limited statistical power of testing but nonetheless patterns can be seen. Increased sample sizes and optimization of feeding schedules post irradiation could possible show further more subtle effects of the MDS.

2.6 Conclusion

Novel object recognition was improved by feeding MDS post-irradiation at both low (5Gy) and high (10Gy) doses. Irradiation groups (5Gy and 10Gy) showed poor performance as expected. The scatter dose appeared to be acting hormetically and also improved novel object recognition. This trend was supported by results of novel object placement testing. Feeding MDS post-irradiation improved recognition of novel placement.

ROS levels are associated with increased anxiety (Bercik et al., 2010) and changes in behaviour (Bouayed, Rammal, & Soulimani, 2009). ROS levels and inflammation seem to be cofounding (Dröge, 2002). Chronic inflammation, as indicated by increased levels of inflammatory cytokines induces anxiety in mice (Bercik et al., 2010). Inflammation can interfere with hippocampal based learning and memory (Czerniawski & Guzowski, 2014). Antioxidant, such as those present in our MDS may improve outcomes.

At the 30 day time mark pro-inflammatory cytokine profile of phase one MDS only fed mice were elevated. This elevated cytokine profile may explain the lack of MDS provided benefit in phase one mice. Inflammation could very well be fueling anxiety avoidance behaviors. Mice fed MDS post-irradiation had an overall decreased and balanced cytokine profile. This supports the increased novelty seeking behaviour seen in

this group during both the NOR and NPR tests. Additional time points would be beneficial to give a more complete picture of MDS interactions since at day 2 after irradiation the MDS only group had low levels of inflammation. Effective ameliorization of deleterious effects of radiation would provide key quality of life benefits in clinical settings which warrants further study of multiple ingredient interventions

CHAPTER THREE:

EFFECTS OF MDA ON OLFACTORY STEM CELLS IN GAMMA IRRADIATED MICE

3.1 Introduction

Olfactory ability is an important social and survival asset to the wild mouse. A keen sense of smell is involved in foraging, kin recognition, selection of a mate, maternal care choices, and even predator avoidance (Brennan & Keverne, 2004). Rodents have two types of chemosensory epithelia in the nasal cavity. The posterior dorsal area of the nasal cavity contains olfactory chemosensory receptors which are more general and are particularly useful for distinguishing volatile odours. An accessory olfactory system, the vomeronasal, is located in the anterior of the ventral floor of the nasal cavity. The accessory olfactory system responds strongly to pheromones but can also help distinguish identity between rodents. Both systems are relevant and offer overlapping functions (Restrepo, Arellano, Oliva, Schaefer, & Lin, 2004).

There are three areas of the brain associated with life-long neurogenesis. The dentate gyrus of the hippocampus is supplied new neurons by the subgranular zone. The olfactory bulb is supplied new neurons from the subventricular zone. The olfactory bulb also contains excitatory axons originating in the olfactory neuroepithelia (Brann & Firestein, 2014). Neurogenesis is necessary for continued olfactory function (Díaz et al., 2011). Neurogenesis must occur after injury, such as injury caused by irradiation. Globose basal cells (GBC) are considered the likely initiators of recovery after mild

injury and horizontal basal cells (HBC) are considered the primary actors after serious injury. Both GBCs and HBCs are multipotent and involved in healing (Brann & Firestein, 2014).

The buried food test was developed as early at the 1970's and has become a standard test for anosmia (Alberts & Galef, 1971). Chemofog impacts the ability to concentrate and attention span (Mitchell & Turton, 2011). Testing olfaction with attractant scents, a highly motivated task, is valuable in order to ensure that olfactory ability is actually tested.

Olfaction is a vital clinical indicator and has well established connections to neurodegenerative disorders such as Dementia and Alzheimer's disease. Stem cell impacts are implicated in cognitive deficits experienced by radiation patients. Measures of olfaction function as indicators of stem cell function.

3.2 Methods

Animals.

Both C57Bl/6J and Tg (Thy1-EGFP) MJrs/J mice (Thy1-GFP) were kept. C57 mice are the background strain for GFP mice with only a green fluorescent protein gene expressed in the brain. All animal care complied with Animal Research Ethics Board's approved Animal Utilization Protocol. Food and water was provided *ad libitum* and mice were housed individually. They were exposed to a 12hr/12hr day/night photoperiod.

Feeding.

The multiple ingredient dietary supplement was mixed fresh every week or frozen for short periods of time. MDS contains 30 different compounds readily available over

the counter. Doses were calculated based on average recommended dosages for humans, adjusted for the smaller weight of a mouse and then increased 10 fold because of a mouse's much higher metabolic rate. MDS was provided on dried bagel bits, a crunchy food favoured by mice, and eaten completely. Consumption of MDS, consumption of standard food, and body weight was monitored to ensure health and that no diet restriction took place. Mice that would receive diet were briefly habituated to MDS prior to the beginning of the trial.

Groups.

Fifty Mice were split into six groups of 5-10 mice at random. Mice were between the ages of 9-11 weeks and each mouse was sampled once. One month after initiation of the diet, irradiation was performed.

Irradiation.

Gamma radiation, produced by a cesium source, was administered to irradiation groups. Mice not scheduled for irradiation underwent sham irradiation. Mice were immobilized for the duration by plastic cones that restricted rotation around the transverse plane. Lead shielding was designed to expose only the head of each mouse.

Olfaction.

Retention of basic olfactory sense was tested in C57 and GFP mice as adapted from procedures describe by Yang and Crawley (2009). Testing arena was an empty mouse cage empty of everything but a 4cm thick layer of woodchips. Mice were habituated to the arena for 5 minutes after which they were removed for 1 minute. During this time a peanut butter scent lure was buried along one wall approximately 1 half inch

beneath the wood chips and the surface of the wood chip bedding was smoothed. Upon re-entry of the arena the time to retrieve the peanut butter lure was recorded. "Failure" was recorded after 15 minutes without finding the lure and the mouse was removed from the test cage. A time cap of 500 seconds was imposed during analysis. Fresh lures and wood shaving were used for each mouse and mice were tested only once.

Statistics.

Group size ranged from 5-12 male mice. Analysis of Variance was performed and Duncan's multiple range test with a criterion of $p<0.5$ was used to determine significance.

3.3 Results

Olfaction.

Control mice took nearly 200 seconds to find a peanut butter lure. MDS mice took only 78 seconds to find the treat which was much quicker than the control but the difference from the control was not statistically significant. Mice that received 10Gy of radiation were slower than both MDS+10Gy+MDS and 10Gy+MDS groups to find the lure (Fig. 34). The 10Gy group had the highest failure rate with 43% of mice failing to find the lure.

3.4 Figures

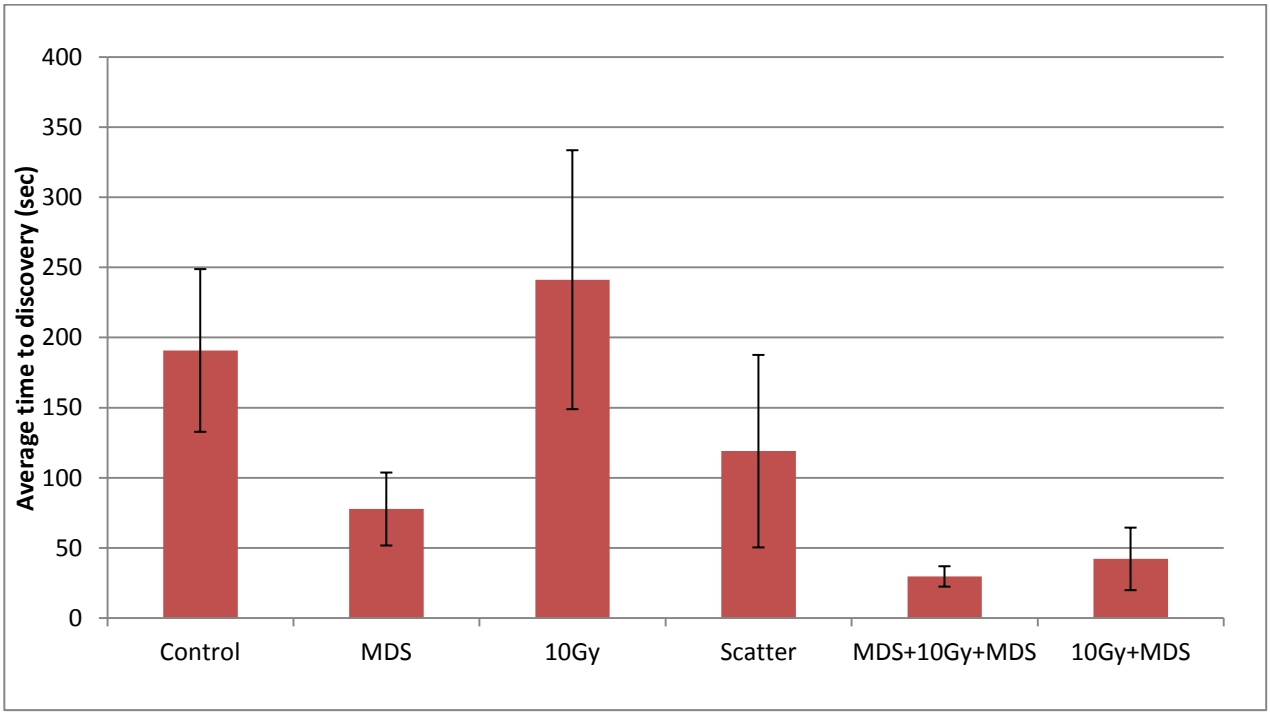

Figure 34: Latency to uncover food in buried food test expressed as the average time that mice in each treatment group took to reach a lure. Lower times indicate a quicker response time to olfactory cues and indicate high olfactory ability. Duncan's multiple range test indicated the 10Gy group was significantly slower to reach the lure than the MDS+10Gy+MDS group ($p<0.03$) and the 10Gy+MDS group ($p<0.04$).

3.5 Discussion and Conclusions

This test checks for anosmia or indicates a basic ability to smell using a high reward peanut butter treat and allows for basic determination of olfactory ability. Changes in olfactory ability has been associated with a decreased olfactory bulb volume associated with oligodendrocyte precursor death (Díaz et al., 2011).

Since testing occurred with peanut butter, a high reward food source, decreased motivation may be less of an issue than for other tests. Since this test took place in a standard housing cage with wood chips the setting would have been much less novel and potentially less anxiety inducing than the white-lined Plexiglas box of the NOR and NPR testing.

Control mice were able to find the lure in less than 200 seconds indicating that the testing conditions were a viable test of rodent olfaction. Mice often burrow or dig when stressed or anxious. Discovery of the lure due to chance uncovering during spontaneous digging would not indicate olfactory ability (Yang & Crawley, 2009). Mice tend to dig most frequently in corners (unpublished observation), so placement of the peanut butter lure alongside the flat surface of the wall acted to decrease the likelihood that the lure was found due to nervous digging. During testing several mice failed to find the lure indicating that the test difficulty was enough to show a distribution of scores based on ability, not chance.

MDS fed groups displayed an increase in targeted exploratory behaviour and resulted in quicker object retrieval during olfaction testing. Scatter dose of 0.5 may have functioned hormetically allowing for improved function over the control. C57 mice

receiving MDS post irradiation had diet compliance decreased by radiation induced nausea. Positive supplementation post-irradiation effects may have been even larger with greater diet compliance.

CHAPTER 4:

DNA/RNA OXIDATIVE DAMAGE

4.1 Introduction

Oxidative stress is linked to many pathologies and neurological related disorders. Oxidative status is critical to apoptosis, DNA damage, inflammation, and mitochondrial function (Y. Hayashi et al., 2008). Oxidative stress is created by Reactive Oxygen Species (ROS).

Many ROS are produced in mitochondria as a by-product of normal respiration. ROS are maintained in a healthy homeostasis by the cell but excess ROS can damage proteins, fatty acids and DNA/RNA (Y. Hayashi et al., 2008). Damaging agents, such as radiation, can cause increased levels of 8-OHdG in tissues. This increase is quickly regulated and repaired by the damaged cells leading to a very short half-life of damage. Hamilton and colleges measured a half-life of only 11 minutes for gamma irradiated tissues in vivo (2001).

ROS attack DNA and RNA molecules and cause replication errors, strand breaks and base damage. There are over 20 different DNA oxidative base adducts (Cooke, Evans, Dizdaroglu, & Lunec, 2003). Guanine is predominately oxidised to 8-hydroxyguanine (8-OHdG) and this adduct is often used as the primary biomarker for oxidative damage (Dizdaroglu, Jaruga, Birincioglu, & Rodriguez, 2002). 8-OHdG is one of the most studies oxidative base adducts. This base damage has been used to estimate oxidative stress and damage from exposure to asbestos, tobacco, and heavy metals and is even used as a biomarker for increased cancer risk (Valavanidis, Vlachogianni, &

Fiotakis, 2009). Mice with high oxidative stress diets (fed deoxycholic acid which increases oxidative stress) developed colon cancer that showed similar morphology to human colon cancers formed in patients with high fat diets. High levels of 8-OHdG were found in surrounding crypt cells and colonic epithelium.

High oxidation status causes damage and this damage has been linked with ageing processes. Neurodegenerative disorders such as dementia and Alzheimer's disease are also strongly linked to oxidative damage. Oxidative damage is measurable in multiple brain regions even in early stages of cognitive impairment leading to Alzheimer's disease (Wang, Markesbery, & Lovell, 2006).

4.2 Methods

Animals.

Mice were individually housed and provided *ad libitum* access to food and water. Care was provided as approved by the Animal Research Ethics Board and outlined in the Animal Utilization Protocol. They were exposed to a 12hr/12hr day/night photoperiod.

Groups. Mice were assigned at random to trial groups upon arrival. Seventy phase one mice were split into 5 groups. Forty phase two mice were split into 6 groups.

Multiple Ingredient Dietary Supplementation.

A dietary supplement was originally designed to ameliorate five major mechanism implicated in aging (Jennifer A. Lemon, Boreham, & Rollo, 2005). Doses were developed based on recommended doses for adults and adjusted for the smaller body mass of a mouse. Doses were also adjusted to account for the higher metabolic rate of mice. MDS

was prepared weekly and either delivered fresh or briefly frozen. MDS was delivered via bagel pieces which were quickly eaten by the mice after a brief adjustment period.

Kit.

Brain tissue homogenate was analyzed using Cayman Chemical's competitive enzyme immunoassay for DNA/RNA oxidative damage.

Statistics.

Groups consisted of 15 male mice. Analysis of Variance was performed and Duncan's multiple range test with a criterion of $p<0.5$ was used to determine significance. There was no significant difference between time points and the data was pooled.

4.3 Results

The group 5Gy+MDS had significantly lower levels of 8-OHdG than the control group and 5Gy group. The MDS only group and the MDS+5Gy+MDS group were lower than the control (MDS $p<0.09$; MDS+5Gy+MDS $p<0.1$) and 5Gy groups but this difference did not reach significance (Fig 35).

4.4 Figure

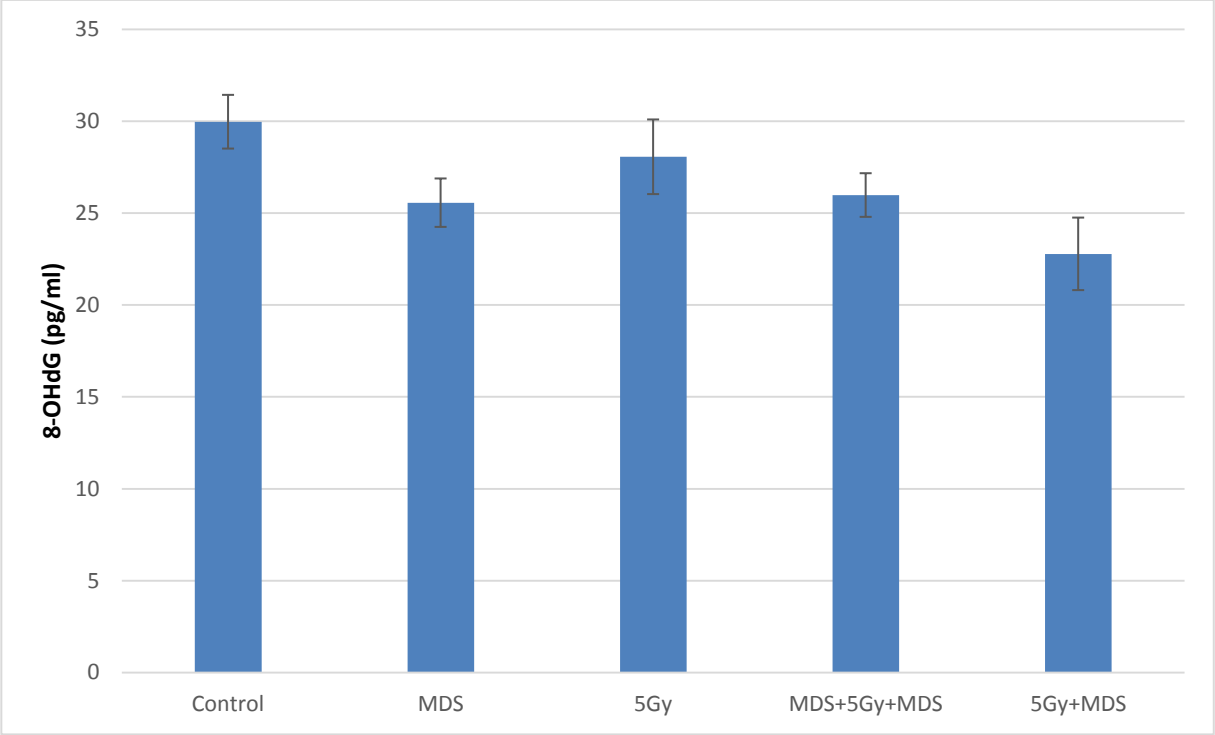

Figure 35: Concentration of 8-OHdG in brain samples of C57 mice. Duncan's multiple range test indicated a difference between the 5Gy+MDS group and the control group ($p<0.006$) and 5Gy group ($p<0.038$). MDS strongly trended towards a lowered 8-OHdG level compared to the control ($p<0.09$) and MDS+5Gy+MDS displayed the same strong trend towards difference from the control ($p<0.1$).

4.5 Discussion and Conclusions

Decreased base damage was shown in the group receiving MDS after irradiation when compared to the control and 5Gy groups. Levels of damage in the MDS alone and continuously fed MDS groups averaged lower than the control and 5Gy groups but were just shy of statistical significance. Increased radiation dosage may further clarify this effect of the MDS.

Results in this biomarker may add clarity to the results of Ch. 4. On day two mice in group 5Gy+MDS had decreased levels of pro-inflammatory cytokines and high levels of anti-inflammatory cytokines. Results of 8-OHdG indicate that this cytokine result may be explained by a prompt resolution of oxidative damage. The results at the 30 day time point for phase one mice contained a confusing similarity of cytokine expression levels between the irradiated group and the group fed post-irradiation. Levels of oxidative damage marker 8-OHdG indicate that low levels of expression the group fed post-irradiation can be attributed to a decreased need for inflammation. Decreased levels of cytokine expression in the irradiated group must be ascribed to another cause, perhaps damage to or decreased ability of the immune system to produce cytokine signals as discussed in Ch. 2. Considering a link between oxidative damage and cytokine expression prompt further exploration into 8-OHdG since there were beneficial results seen in cytokine expression in mice fed the diet continuously at day 120. Larger group sizes at each time point (n equalled 5) may reveal subtle differences between time points that this study design was unable to capture.

Decreased transcription of mitochondrial RNA and decreased copy numbers of mitochondrial DNA are additional measures of oxidative damage. Pre-treatment with melatonin has been shown to decrease these types of damage (Xu et al., 2010). Diet modifications have been shown to reduce damage and even protect leukocytes (Chen et al., 2001).

CONCLUSIONS

Inflammation.

CDS has a complex effect on inflammation status. At the early time point of 2 days, continuous MDS appears to have protected against pro-inflammatory reactions but has also blocked strong anti-inflammatory signals. This trend continues for the lower dose of 5Gy without spikes in cytokine expression. Feeding post-irradiation seems to boost immune function and produce a strong acute response to damage caused by irradiation but over time restores normal levels of cytokine expression. At 120 days the impacts of feeding continuously or only post-irradiation seem to be similar.

After a higher dose of 10Gy, pro-inflammatory and anti-inflammatory cytokines were able to climb in the continuously fed irradiation group. The MDS diet may have a limited ability to block damage and cytokine signalling and 10Gy may overwhelm its protective effects. Feeding post-irradiation seemed to allow larger acute pro-inflammatory and anti-inflammatory responses but at 30 days offered a more reduced cytokine levels than continuous feeding. MDS appears to offer both protective effects and the ability to ameliorate damage. Cycling MDS administration may avoid atrophy of body defenses while still offering boost in immune function after damage.

Novelty.

NOR. MDS alone and continuously feeding MDS did not provide significant benefits in NOR. Inflammation markers indicate that the MDS post-irradiation group had the lowest levels of systemic inflammation at 30 days and normal levels at 120 days and this group performed the best. Phase two mice had similar results, with the MDS post-irradiation

group performing well. The scatter dose also performed well and this dose may have been acting hormetically.

NPR. Results in phase two mice support that of NOR. Less benefit is seen in the scatter dose group but the MDS post-irradiation group is still the best performing group.

ROS. Measures of ROS should be measured to give a better idea of the mechanisms behind these novelty seeking or anxiety driven behaviours.

Olfaction.

Results in olfactory testing point towards interesting beneficial effects of the MDS for stem cells. Protection of stem cell function is important for olfaction and hippocampus dependent functions (Imayoshi et al., 2008). Olfaction acts as an early indicator of compromised stem cell function (Brann & Firestein, 2014). Decreases in olfaction have been linked to diseases such as Parkinson's and Alzheimer's.

8-OHdG.

Levels of 8-OHdG added some clarity to cytokine results. Anti-inflammatory cytokine expression on day two were high indicating a timely resolution of inflammation causing damage. Cytokine expression in the group fed post irradiation was low across the board on day 30 while levels of 8-OHdG were low indicating that low cytokine levels were due to decreased need for damage repair.

Overall levels of indicated oxidative DNA/RNA damage match closely with results of behavioural testing (Ch. 2) and sensory abilities (Ch. 3). Lower levels of damage seen in the group fed MDS post-irradiation was correlated with improved

performance. MDS alone and MDS fed continuously acted beneficially but feeding MDS post-irradiation proved most significantly beneficial with lower levels of damage.

Future Directions.

This compound could provide significant quality of life improvement for cancer survivors. Since the timing of diet administration appears to be crucial further studies are warranted. A modified administration schedule that cycles between high and low doses may be beneficial. This schedule structure could help prevent atrophy of defense or repair systems while still augmenting their function.

Appendix A

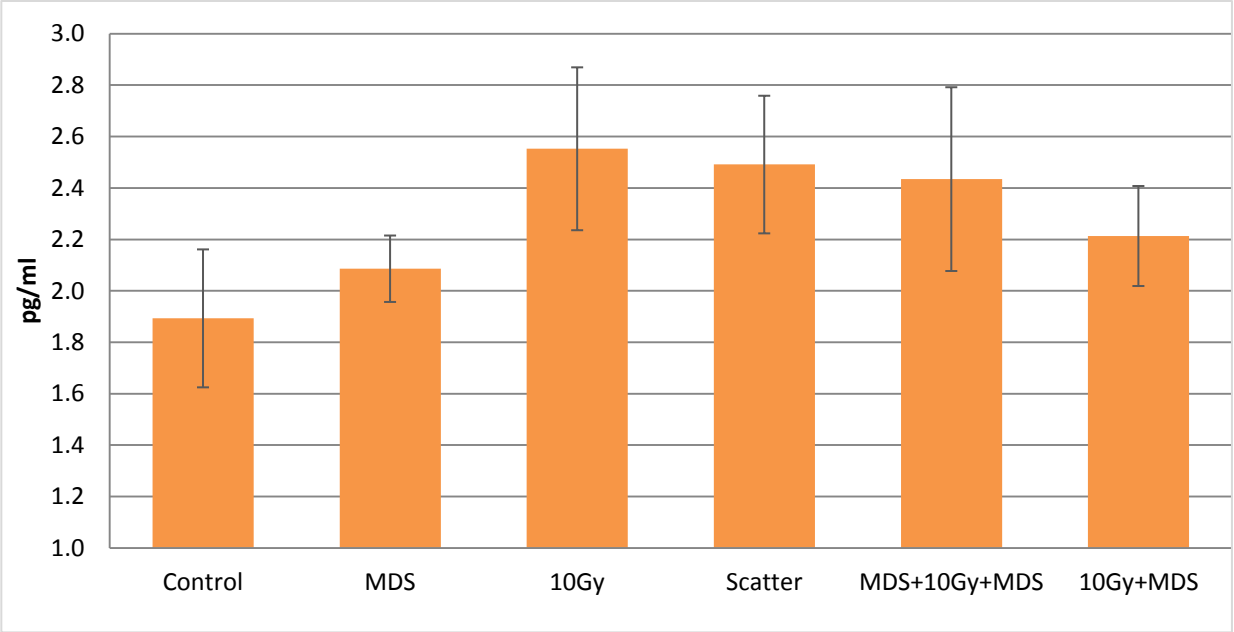

Figure 36: Effects of various treatments on circulating levels of TNF-α in pg/ml at 30 days in phase two mice. Analysis of variance indicated no significant differences between groups.

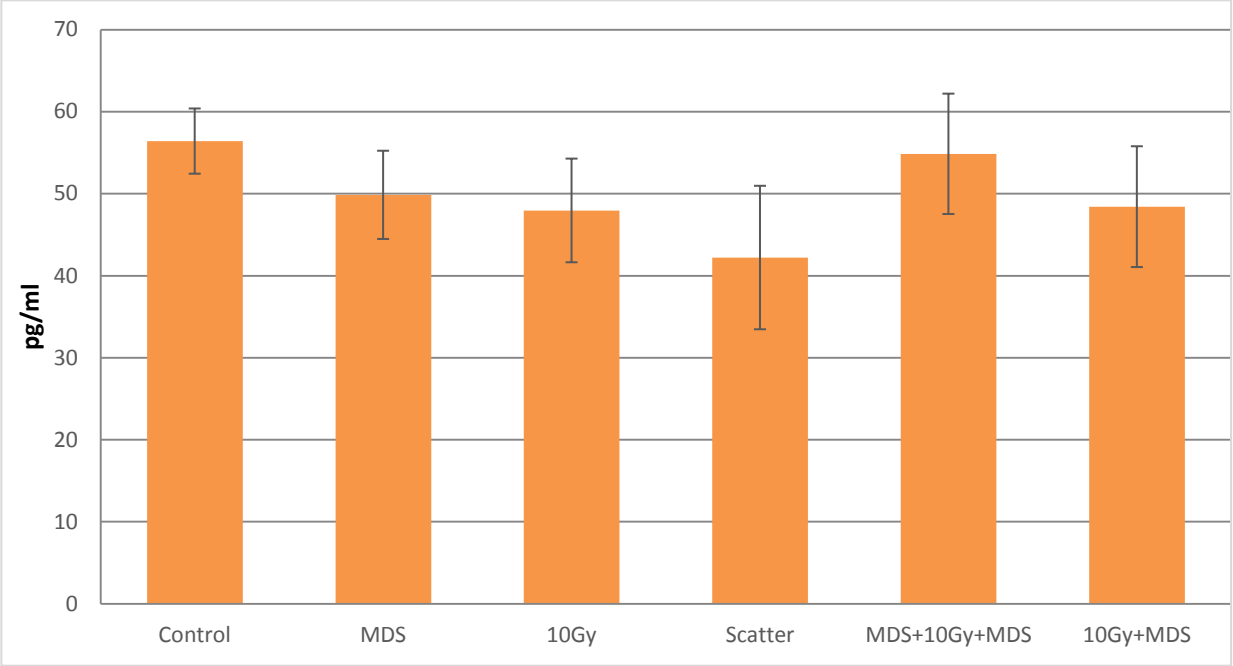

Figure 37: Effects of various treatments on circulating levels of IL-12p70 in pg/ml at 30 days in phase two mice. Analysis of variance indicated no significant differences between groups.

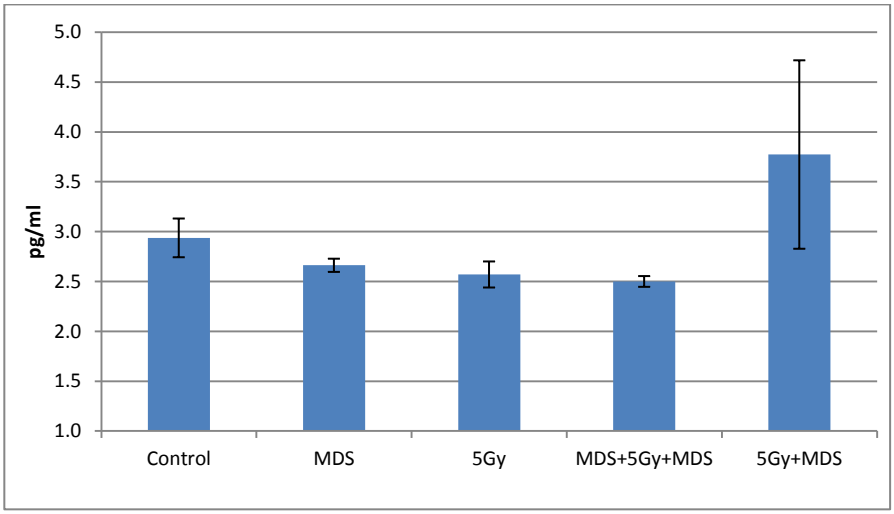

Figure 38: Effects of various treatments on circulating levels of IL-2 in pg/ml at 120 days. Analysis of variance indicated no significant differences between groups.

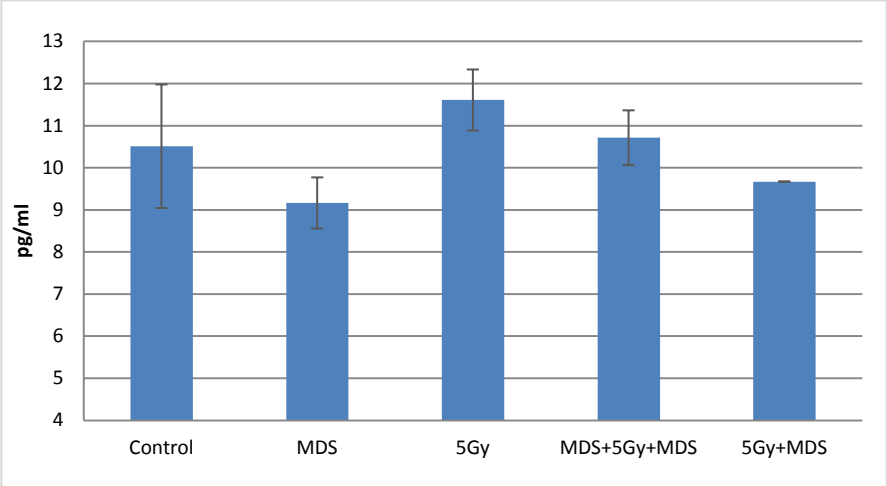

Figure 39: Effects of various treatments on circulating levels of IL-10 in pg/ml at 120 days. Analysis of variance indicated no significant differences between groups.

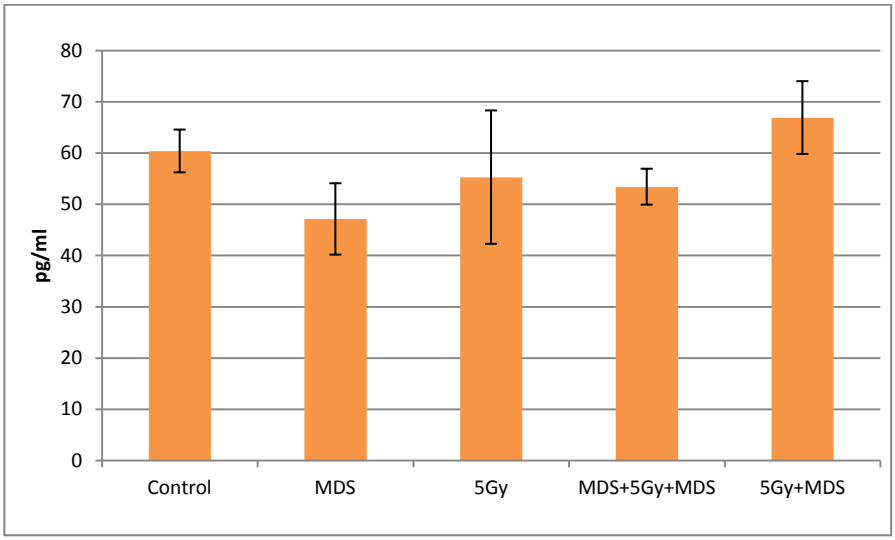

Figure 40: Effects of various treatments on circulating levels of IL-12p70 in pg/ml at 120 days. Analysis of variance indicated no significant differences between groups.

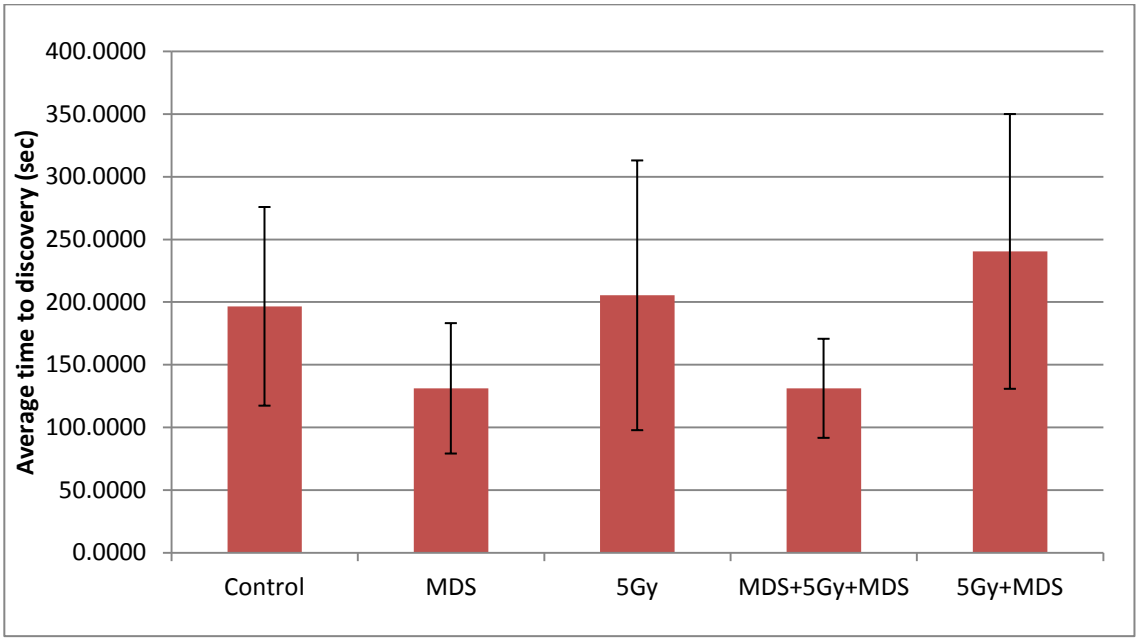

Figure 41: Latency to uncover food in buried food test expressed as the average time that C57 phase one mice in each treatment group took to reach a lure. Lower times indicate a quicker response time to olfactory cues and indicate high olfactory ability. Analysis of Variance indicated no significant between groups. Trends shown above follow the trends of GFP mice tested and reported in Ch. 3.

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