THE REVERSAL OF LOW LEVEL GAMMA IRRADIATION INDUCED ABERRATIONS IN THE RAT TESTES: A HISTOLOGICAL, ENDOCRINOLOGICAL AND SPERM KINEMATIC EVALUATION

BY

P. L. MABETA

Submitted in partial fulfillment of the requirements for the degree of Master of Science in the department of Physiology in the Faculty of Veterinary Science, University of Pretoria

Date submitted: JUNE 2002
DECLARATION

I, PEACEFUL MABETA, HEREBY DECLARE THAT THE WORK ON WHICH THIS THESIS IS BASED WAS CARRIED OUT BY ME. I ALSO DECLARE THAT NO PART OF THIS WORK HAS BEEN, IS BEING, OR IS TO BE SUBMITTED FOR ANOTHER DEGREE AT THIS OR ANY OTHER UNIVERSITY.

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ACKNOWLEDGEMENTS

‘Interdependence is a higher value than independence’ Stephen Covey

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THE REVERSAL OF LOW LEVEL GAMMA IRRADIATION INDUCED ABERRATIONS IN THE RAT TESTES: A HISTOLOGICAL, ENDOCRINOLOGICAL AND SPERM KINEMATIC EVALUATION

By

P. L. MABETA

PROMOTER : DR J. M. BRINDERS
CO-PROMOTERS : PROF N. C. BENNETT
PROF J. G. VAN DER WALT
DEGREE: MSc
DEPARTMENT: VETERINARY PHYSIOLOGY

SUMMARY

Radiotherapy for cancer produces prolonged testicular damage that is manifested by failure of spermatogenic recovery following treatment. It has thus become important to develop methods to induce the recovery of spermatogenesis, if fertility is to be restored in patients (De Vita et al., 1997). The importance of steroid hormones in the control of testicular function has lead to numerous studies being conducted on the use of these hormones as treatment to protect the testes from radiation damage (Desjardins and Ewing, 1993; Kurdoglu et al, 1994; Kangasniemi et al., 1996a; Shuttlesworth et al., 2000).
The aim of this study was to investigate the effects of low dose gamma irradiation on the rat testes and the use of testosterone to reverse damage to the testes resulting from radiation exposure.

Sprague Dawley rats were subjected to irradiation doses of 3.5 and 6.0 Gy. The rats were treated with testosterone over 4 and 8 weeks. Analyses were carried out at three levels: histological, kinematic, and endocrine.

Irradiation led to a dose dependent reduction in spermatogenic cell numbers. Percentage sperm motility was decreased, and two of the five measured kinematic parameters, curvilinear velocity and straight line velocity, were decreased. Luteinising hormone (LH) and follicle stimulating hormone (FSH) concentrations increased in a dose dependent manner, while testosterone concentration showed insignificant fluctuations.

Following testosterone administration, spermatogenic cell numbers improved. LH concentrations were restored to almost control levels.

Testosterone administered following exposure of the rat testes to low dose gamma irradiation led to the recovery of spermatogenesis.
CHAPTER 1: INTRODUCTION

1.1. HYPOTHESIS

Extensive research has been conducted on the effects of irradiating the testes (Kurdoglu et al., 1994; Kangasniemi et al., 1996b; Wang, et al., 1993; Meistrich and van Beek, 1993) and exposure of the testes to irradiation leads to impaired fertility (Morris and Shalet, 1990; Wang et al, 1993). More than 20 000 males of reproductive age per annum receive radiotherapy alone or in combination with chemotherapy for the cancers mentioned above (Silverberg et al., 1990; Wilson et al., 1999). This form of treatment has produced a success rate of 80% (Velasquez et al., 1991). Nonetheless, patients receiving this treatment become azoospermic, and of the few patients who recover from azoospermia, the sperm count only reaches oligospermic levels (Shamberger et al., 1981; Kurdoglu et al., 1994).

As a result, during the 1980’s research directed at finding ways to minimise the effects of irradiation on the testis was undertaken, with the explicit aim of developing treatment for patients receiving radiotherapy (Blanchard and Boekelheide, 1998).

Studies conducted on rats have shown that the administration of hormones, and in particular testosterone, prior to exposure to low dose (below 7.0 Gy) irradiation enhanced recovery of spermatogenesis (Kurdoglu et al., 1994; Kangasniemi et al., 1996a). Today pre-hormone treatment, in particular GnRH agonists Lupron (leuprolide acetate, TAP Pharmaceutical Inc. IL, USA) and Zoladex (goserelin acetate, Zeneca pharmaceuticals, Delaware), are administered prior to radiation therapy in patients treated for prostate cancer (Correspondence, Iowa University Hospital).
The major disadvantage of such treatment in cancer patients is that hormones must be administered 12 weeks prior to the commencement of radiation treatment (time needed for complete suppression of spermatogenesis in man).

It is well known that to attain the best results in cancer treatment, therapy must be started in the early stages of the disease. Testosterone was employed in this study to stimulate spermatogenic recovery following irradiation of the testes.

The first chapter provides a general overview of the control of testicular function. The effects of irradiation on the testis and the treatment modalities adopted to date to minimize these effects and their shortcomings are discussed.
1.2. LITERATURE REVIEW

1.2.1. RADIATION

Radiation is the emission or transfer of radiant energy in the form of waves or particles (Prasad, 1984). Radiation comprises two types: ionizing and non-ionizing radiation. Ionizing radiation is radiation emitted by radionuclides, which are elements in an unstable form (Prasad, 1984). It can cause structural changes by removing electrons from atoms and leaving behind a charged atom (ion).

Non-ionizing radiation does not result in structural changes of atoms (it does not cause ionization). Non-ionizing includes radiation from light, radiowaves, and microwaves. This study deals with the effects of ionizing radiation. Therefore the term radiation will be used to denote ionizing radiation.

All ionizing radiation interacts with living matter in a similar way. However, various types of radiation differ in their effectiveness in damaging a biological system. To determine this effect, the term ‘Relative biological effect’ (RBE) was coined (Altman and Lett, 1990).

1.2.2. RELATIVE BIOLOGICAL EFFECT

Relative biological effect (RBE) is the measure of damage produced by the same dose of different radiations (Prasad, 1984). RBE has been used to assess the damage caused by gamma-, X-ray, and neutron irradiation on the testes (Wilson et al., 1999).
There are differences in the rate at which matter is ionized by different types of radiation. These differences have lead to the development of the concept of linear energy transfer (LET) (Altman and Lett, 1990). The concepts of ‘RBE’ and ‘LET’ are introduced later in the discussion on mechanisms of hormone therapy.

LET is defined as the energy deposit per path length of the original particle (Altman and Lett, 1990). The RBE of radiation increases with increasing LET. However, beyond a certain point (maximum), an increase in irradiation dose (or LET) does not cause further damage, and RBE remains unchanged. Gamma rays are an example of low-LET radiation, while neutron radiation is high-LET. Various units have been adopted to express the quantity of radiation.

1.2.3. UNITS OF RADIATION

The quantity of absorbed radiation was initially measured in roentgens (Prasad, 1984). Subsequently, it was measured in rads (radiation absorbed dose), where 1 roentgen equals 0.93 rad. The rad has been recently replaced by the gray (Gy). One Gy equals 100 rads (Potten and Henry, 1983). In this study the gray was chosen as the unit to express the dose of irradiation. The Gy is the most widely cited unit in currently reviewed literature.
1.2.4. BIOLOGICAL EFFECTS OF RADIATION

The effects of irradiation on biological matter have been studied extensively. Mettler and Mosley (1978) published a comprehensive account of how radiation interacts with biological matter. According to Mettler and Mosley (1978), when a cell or tissue is exposed to radiation, several changes occur, e.g.

a). damage to the cell membrane through lipid peroxidation
b). damage to either one or both strands of deoxyribonucleic acid (DNA)
c). formation of free radicals causing secondary damage to cells

Considering the above, radiation can lead to damage in two ways:

First, directly by breaking molecular bonds of cellular components. This occurs when radioactive particles impact upon a compound or cellular component.

Secondly, indirectly via the formation of free radicals (highly reactive atoms or molecules with a single unpaired electron). These radicals may either inactivate cellular mechanisms or interact with the genetic material (DNA).

The damage caused by radiation can be repaired, or the cell may survive and multiply in a deformed manner (Mettler and Mosley, 1978). Figure 1 below represents a brief but concise illustration of the changes a cell may undergo following exposure to radiation.
Mettler and Moseley (1978) suggest that if only one strand of DNA is broken by ionising radiation, repair could occur within minutes. However, when both strands of DNA are broken at about the same position, correction would be much less likely (Mettler and Moseley, 1978). But if there are a large number of ionizations in a small area (more than one single break in the DNA strand), the local cell repair mechanisms become overwhelmed. In such cases the breaks in DNA may go un repaired, leading to cell mutations (Altman and Lett, 1990).

1.2. 5. EFFECTS OF IRRADIATION ON THE GONADS
The effects of irradiation on gonadal tissue have been studied extensively in both man and rodents (Cunningham and Huckins, 1978; Erickson, 1978; Delic et al, 1987; Kurdoglu et al, 1994; Kangasniemi et al., 1996).
Generally, the effects of irradiation upon the male reproductive system include a gradual loss in testis mass (Cunningham and Huckins, 1978; Erickson, 1978) and decreased weight of the accessory organs (Birioukov et al., 1993). A decrease in testicular blood flow has also been observed in some studies (Wang et al., 1993). The most pronounced and widely cited effect of irradiation on the testes, however, is the elimination of differentiating spermatogonia, followed by a depletion of more advanced spermatogenic cells (Kurdoglu et al., 1994; Kangasniemi et al., 1996; Shuttlesworth et al., 2000). Desquamation and vacuolization of germinal cells, and multinucleated giant cells have also been observed (Matsumiya et al., 1999). According to Matsumiya et al. (1999), the extent of the damage appears to be dose dependent.

Unlike albino rats, humans and Sprague Dawley rats are rendered azoospermic by doses in the order of 2-3 Gy (Kurdoglu et al., 1994). Dosages above 6.0 Gy destroy all classes of spermatogenic cells, except non-dividing stem cells. Recovery of spermatogenesis is not observed without hormone intervention (Shuttlesworth, 2000).

Prior to discussing the hormone treatment modalities employed in different studies, a brief physiology of testosterone is reviewed.

1. 2. 6. TESTOSTERONE

Testosterone is the principal hormone of the testis. It is a C19 steroid with a hydroxyl group at C17, and is synthesized by cholesterol in the Leydig cells (Steinberger et al., 1973).
i). SYNTHESIS OF TESTOSTERONE

The secretion of testosterone is under the control of Luteinizing hormone (LH). LH acts via serpentine receptors to increase the level of cyclic adenosine monophosphate (cAMP) in the Leydig cells (Yen and Jaffe, 1978). Current evidence supports the notion that the mechanism by which LH evokes steroid synthesis depends upon minute increases in cAMP production, leading to increased protein kinase activity (Yen and Jaffe, 1978). This, in turn, leads to the activation of 20,22 desmolase. The compound 20,22 desmolase catalyses the side chain cleavage of cholesterol to pregnenolone. The conversion of cholesterol to pregnenolone is the first and committed step in the synthesis of testosterone (Murray et al., 1993). Following secretion, testosterone is transported in the circulation in various forms.

ii). TRANSPORT

Ninety eight percent of the testosterone in the blood is transported bound to plasma proteins: of this 65% is bound to sex steroid binding globulin, and the remaining 33% to albumin (Yen and Jaffe, 1978). In contrast, only 2% of the total plasma testosterone is transported as a free form (Yen and Jaffe, 1978).

Free testosterone enters the cells through the plasma membrane either passively or through facilitated diffusion (it is a steroid hormone). Testosterone is retained by target cells, because it associates with a specific intracellular receptor once inside the cell (Yen and Jaffe, 1978). The receptor steroid complex then binds to DNA in the nucleus, facilitating the transcription of various genes.
The cytoplasm of some target cells contains the enzyme 5α-reductase, which converts testosterone to dihydrotestosterone (DHT). These target cells include the Sertoli cells, the prostate gland, the seminal vesicles, external genitalia, and the genital skin. Whether DHT acts via different receptors to those of testosterone in these organs has been a point of controversy, but the consensus is that while there is a single class of receptors, there is greater receptor affinity for DHT than for testosterone. This affinity difference, coupled to the ability of a target tissue to form DHT from testosterone, may determine whether the testosterone-receptor complex or the DHT-receptor complex is active (Yen and Jaffe, 1978).

Testosterone may also be converted to oestrogen by the enzyme aromatase in the cytoplasm of some cells. Cells containing this enzyme include the brain cells, the Sertoli cells of the immature male, and Leydig cells in the adult male (Janulis et al., 1998). Testosterone is metabolised in most tissues to produce 17-ketosteroids, mainly androsterone and etiocholanolone. In the liver these ketosteroids are conjugated with glucuronide and sulphate to make them water-soluble. These end products are then excreted via the kidneys (Yen and Jaffe, 1978).

The importance of gonadotropic and steroid hormones (in particular testosterone) in the control of testicular function has lead to a number of studies on hormonal treatments in order to protect or induce recovery of spermatogenesis following radiation exposure (Morris, 1993).
1.2.7. EFFECTS OF EXOGENOUS TESTOSTERONE

Generally, exogenous testosterone results in increased plasma testosterone levels. High levels of circulating testosterone lowers the level of LH manufactured and released in the pituitary by suppressing its production by the gonadotrophs. Reduced circulating LH leads to a subsequent reduction in testosterone production by the Leydig cells. The levels of intratesticular testosterone (ITT) also decreases. For instance, testosterone administered at 0.7 mg/kg suppresses ITT in rats (because it suppresses endogenous testosterone production). Sperm count has also been shown to decrease following exogenous testosterone administration (Kurdoglu et al., 1994).

1. 2. 8. HORMONE THERAPY

Sterility in males is a frequent side effect of radiotherapy administration for cancer. As a result, several regimens have been adopted in various studies to minimize the effects of irradiation upon the testes.

The protective hormonal treatments given prior to and during cytotoxic treatment include, GnRH-agonists (Meistrich et al., 1997), GnRH-antagonists (Matsumiya et al., 1999; Shuttlesworth et al., 2000) and GnRH analogues combined with anti-androgens (Kangasniemi et al., 1996a). The majority of studies have employed the rat as a model system since as in man, radiation produces extensive testicular damage, however, recovery takes longer in humans (Kangasniemi et al., 1996a; Meistrich et al., 1996).
Kangasniemi et al. (1996b) employed the GnRH-agonist Zoladex (Goserelin Acetate, Zeneca pharmaceuticals, Delaware) to suppress sperm production and enhance recovery of spermatogenesis in the mouse following exposure to 10.0 Gy irradiation, but recovery was not enhanced by this regimen. The reason cited by Kangasniemi et al. (1996b) was that almost no A spermatogonia were found in 80% of the mice following exposure to 10.0 Gy of gamma irradiation.

Shuttlesworth et al. (2000) used the GnRH antagonist Cetrorelix (cetrorelix pamoate, ASTA Medica, Frankfurt) as a hormone treatment in the rat. Cetrorelix was administered 10 weeks following exposure to 6.0 Gy of irradiation. Decreased levels of intra-testicular testosterone (ITT) were reported.

In the group given Cetrorelix, differentiation of spermatogonia was seen at week 4, and there was 30% re-population of spermatogonia at 6 weeks, whereas in the control group (no Cetrorelix given) no repopulation occurred. Shuttlesworth et al. (2000) proposed that the lowering of ITT following the administration of Cetrorelix might have been responsible for the changes in spermatogonial cell numbers.

Other hormones utilised in studies on enhancing the recovery of spermatogenesis from radioactive exposure include testosterone administered alone (Sharpe et al., 1986) and oestradiol administered in addition to testosterone (Kurdoğlu et al., 1994; Wilson et al., 1999).
1.2.9. TESTOSTERONE TREATMENT

The effects of testosterone and a combination of testosterone and oestradiol treatment prior to exposure to irradiation has been studied by Kurdoglu et al. (1994) and Wilson et al. (1999). Sharpe et al. (1988) administered testosterone at a dose of 25 mg over three days to protect germinal cells prior to irradiation. The ITT levels were 32% lower than that of the control. Testosterone protected the germinal epithelium from radiation damage. A combination of testosterone and oestradiol was used to treat rats prior to irradiation (Kurdoglu et al., 1994).

The rats were given testosterone plus oestradiol 6 weeks prior to radiation with a dose of 3.5 Gy. Recovery took 9 weeks. This treatment resulted in a dose-modifying factor of 2 (i.e. the effect of the treatment was the same as though the rats were subjected to a dose of 1.7 Gy).

Dose modifying factor (DMF) is a parameter adopted in some studies to determine the degree of protection from radiation treatment. DMF is defined as the neutron or gamma ray dose given to animals that were treated with hormones to produce a given effect, divided by the dose necessary to produce that same effect in the control (Wilson et al., 1999).

Wilson et al. (1999) administered testosterone in addition to oestradiol prior to irradiation with 0.7-2.7 Gy of cyclon generated high-energy neutrons. They also assessed recovery of spermatogenesis 9 weeks post irradiation.
Greater recovery was observed (increased testis weight, repopulation indices, and sperm counts) compared to the cholesterol treated control (Wilson et al., 1999). Therefore testosterone administered prior to irradiation, either singly or in combination with oestradiol, enhanced spermatogenic recovery in rats subjected to low dose irradiation.

1.2.10. MECHANISM OF HORMONE ACTION

Based on reviewed literature, three theories have been proposed to explain the hormonal modulation for the recovery of spermatogenesis:

According to the first theory (Glode et al., 1981), hormonal treatment prior to irradiation protects the testis by interrupting the hypothalamo-pituitary-gonadal axis. The disruption may lead to a decrease in the testicular production of testosterone. This in turn would lead to a reduction in the rate of spermatogenesis (less spermatogonia would be stimulated to divide), and hence this would render the spermatogenic cells more resistant to radiation treatment.

Since the target cells for prolonged reduction of spermatogenesis were generally considered to be the spermatogonia, the treatment had been assumed to render spermatogonia quiescent. However Meistrich et al, 1999, showed that hormonal treatment that protects the recovery of spermatogenesis has no effect on spermatogonial kinetics.
A second theory proposed to explain the possible mechanism of protection of the germ cells from irradiation is that of hormone pre-treatment which leads to a reduction of the oxygen tension within the testis, enhancing thiol-mediated protection against radicals, or an enhancement of DNA repair. Wilson et al. (1999) investigated the potential role of these mechanisms. Oxygen increases radiosensitivity by reacting with free radicals on the DNA and forming peroxides, which in turn leads to DNA damage. In contrast, thiols scavenge free radicals in competition with oxygen, thereby reducing the degree of DNA damage and reducing sensitivity to irradiation. According to Wilson et al. (1996) to investigate the validity of this theory, it was necessary to find an agent less toxic and dependent on oxygen, thiols and DNA repair.

Wilson et al. (1996) employed neutron radiation with a high LET and thus less dependent on these factors. Gamma rays represent low LET radiation and dependent on the three factors already mentioned. Neutrons produce more direct and less hydroxyl radical-mediated damage than photons. Thus if the three factors mentioned were involved in the mediation of hormone treatment prior to gamma irradiation exposure, the protective effect against neutron radiation should be lower (Wilson et al, 1999). Based on the findings of Wilson et al. (1999), there was an equal protection factor for both types of radiation.

None of the three factors mentioned above appear to be involved in the mediation of protection of spermatogenesis by hormonal treatment. The rationale given in the third theory is that treatment may stimulate the proliferation of stem cells (Van Alphen et al. 1989). Van Alphen et al. (1989) proposed that proliferating stem cells could be more resistant than quiescent ones.
This theory, however, is contradicted by studies conducted almost a decade later by Meistrich et al. (1999). In their studies they showed that there was no change in the numbers of stem cells or their immediate progeny after suppressive treatment with hormones that resulted in protection on recovery of spermatogenesis.

Testosterone administered prior to irradiation singly or in combination with oestradiol enhances recovery of spermatogenesis in rats exposed to low dose irradiation. Nonetheless, there has been no elucidation of the mechanism by which these hormonal treatments protect the survival of stem spermatogonia. Although the mechanism of the treatment has not satisfactorily or convincingly been elucidated, several authors agree that for hormone treatment to be successful, it should result in the suppression of intra-testicular-testosterone (ITT), a reduction in testicular weight, as well as a decrease in sperm count. Also according to Shuttlesworth et al. (2000), successful induction of recovery of spermatogenesis requires the presence of surviving stem cells.
1.3. AIM OF STUDY

It has been established through the literature that thousands of male patients in their reproductive age undergo radiotherapy for various forms of cancer (Wilson et al., 1996). Although highly effective, radiotherapy leads to azoospermia (Meistrich and Kangasniemi, 1997; Kurdoglu et al., 1994). The present mode of hormone treatment, which is given prior to radiotherapy, compromises patient health due to the waiting period before cancer treatment can be started.

On the other hand, all successful hormone treatments have been shown to work by decreasing ITT levels (Kurdoglu et al., 1994; Meistrich and Kangasniemi, 1997; Wilson et al., 1999; Shuttlesworth et al., 2000). Exogenous testosterone depresses testosterone production by the Leydig cells, and this leads to reduced ITT levels. Therefore, exogenous testosterone might have a role in stimulating recovery of the testes from low dose gamma irradiation.

Furthermore, administering testosterone after radiotherapy would allow cancer treatment to be started immediately after diagnosis, and would not interfere with the course of cancer treatment.

The aim of this study was to determine whether testosterone could reverse the detrimental effects of low dose gamma irradiation on the rat testis if administered following exposure to irradiation. The histological, sperm kinematic, and endocrinological changes following exposure to irradiation were assessed in rats administered and not administered testosterone. The resulting effects of testosterone treatment on these parameters were investigated.
CHAPTER 2: HISTOLOGICAL EVALUATION OF THE EFFECTS OF GAMMA IRRADIATION ON THE TESTES OF SPRAGUE DAWLEY RATS

2.1. INTRODUCTION

The structure of the testis in mammals is characterised by the presence of two functional components, the seminiferous tubules, and the interstitial tissue (Burger and de Kretser, 1981). The interstitial tissue comprises loose connective tissue situated between the tubules. Small collections of Leydig cells are seen in the stroma. Lying at the basement membrane of the seminiferous tubules are Sertoli cells that move germ cells along to the lumen (De Kretser, 1982). They also serve as routes through which nutrients reach the developing spermatogenic cells (De Kretser, 1982).

Spermatogonia, which are the outermost spermatogenic cells found at the basement membrane of the seminiferous tubules, are the least differentiated of spermatogenic cells. These cells are under division almost continually by mitosis (Burger and de Kretser, 1981). The most adopted model for explaining stem cell renewal in rats is that proposed by Huckins and Oakberg (1978). This model assumes the existence of a renewing stem cell line with a very long cell cycle ($A_s$). It also assumes that stem cell renewal occurs by division of some of these $A_s$ cells. The dividing $A_s$ cells form $A_{pr}$ (paired), $A_{al}$ (aligned), $A_1$, $A_2$, $A_3$, $A_4$, In (intermediate), and then B spermatogonia. Each spermatogonium in the clone will then undergo the final mitotic division and differentiation to form a primary spermatocyte.
Primary spermatocytes undergo their first meiotic division to form secondary spermatocytes. Secondary spermatocytes undergo a second meiotic division to form spermatids (Burger and de Kretser, 1981). Finally spermatids differentiate into spermatozoa. It takes 4 cycles to complete maturation. Each cycle takes approximately 13 days (Meistrich and van Beek, 1993).

2.2. EFFECTS OF IRRADIATION ON THE TESTES

At low doses of irradiation, the Sertoli and Leydig cells do not show any histological changes because they are fairly radio resistant (Mettler and Moseley, 1987). At dosages above 10.0 Gy, no morphological changes are seen in these cells, but they start to shrink (Mettler and Mosley, 1987). The differentiating spermatogonial cells (type A and B) are radiosensitive, while spermatocytes, spermatids, and spermatozoa are relatively radio-resistant (Erickson, 1978). Non-differentiating spermatogonial cells, also referred to as stem cells, are the most radioresistant of all spermatogenic cells (Pinon-Lataillade et al., 1991; Kurdoglu et al., 1994; Kangasniemi et al., 1996; Meistrich et al., 1996). It is the division and differentiation of these surviving cells that is responsible for the repopulation of the seminiferous epithelium following exposure to radiation (Shuttlesworth et al., 2000).

The effect of irradiation on spermatogenic cells is dose dependent (Pinon-Lataillade et al., 1985). After radiation with low doses of gamma rays (~0.9-3 Gy), differentiating spermatogonia are killed. The depletion of spermatogonia results in a reduction of subsequent spermatozoa (Pinon-Lataillade et al., 1985; Pinon-Lataillade et al., 1991; Kangasniemi et al., 1996; Meistrich et al., 1996).
However, at doses below 3.0 Gy, the surviving stem cells can repopulate the seminiferous tubules without drug or hormone intervention, although the period of recovery is dependent on the radiation dose (Kangasniemi et al., 1996). Dose levels of between 3.3 and 4 Gy of $^{60}$Co gamma irradiation and above destroys all the classes of differentiating spermatogonial cells (Meistrich, 1998). Following exposure of the testes to 3.5 Gy, repopulation of the spermatogonial cells is not observed for 60 weeks post irradiation (Shuttlesworth et al., 2000). This is despite the presence of type A spermatogonia, which, according to Shuttlesworth et al. (2000), were likely to be stem cells. Stem spermatogonia can survive doses above 6.0 and 7.0 Gy of irradiation (Shuttlesworth et al., 2000).

2.3. DETERMINATION OF RECOVERY OF THE TESTES

Stem cell number is the main parameter affected by exposure of the testes to low dose gamma irradiation. Hence these cells can be used as a suitable parameter for assessing testicular recovery following exposure to irradiation. The clonogenic assay is one method employed to determine the ability of surviving stem cells to differentiate (Meistrich van Beek, 1993). In this assay, colonies of differentiated cells that have reached the type B spermatogonial stage or later are counted (Meistrich van Beek, 1993). Counts of repopulating and non-repopulating seminiferous tubule cross sections are determined several weeks (3-13 weeks in rats and mice) after completion of treatment with a toxin. Theoretically, this time frame should prove optimal for this assay; all colonies should contain at least spermatocytes, and some may contain spermatids, making repopulating tubules cross sections distinguishable from non-repopulating ones (Meistrich and van Beek, 1993).
2. 2. MATERIALS AND METHOD

2. 2. 1. ANIMALS

Male Sprague Dawley rats were purchased from the central animal service at the University of the Witwatersrand (Wits), Johannesburg. The mean body mass (SE) of the rats was 502 g ± 0.06. The rats were housed at the Wits central animal service in a temperature-controlled room (21 °C) with standard lighting conditions (12 L-12 D). The animals were kept in 60 x 40 cm cages (4 rats per cage). They were given standard commercial food and water *ad libitum*. The rats were weighed weekly until the time of sacrifice. The radiation procedure was performed with the assistance of a technician at Wits. The administration of all drugs was performed with the help of a veterinary nurse and a veterinary surgeon. All projects were approved by the Animal Ethics Committees at Wits (project 99-51-4) and the University of Pretoria (project 36-5-402).

The rats were anaesthetised prior to irradiation exposure in order to minimise stress. All rats were anaesthetised using 0.5 mg Anaketamine /kg (Centaur labs, Bryanston). The anaesthetic was injected intra-muscularly (i.m.). Rats in groups A and B were given 0.25 mg Rompun (Bayer, Isando) following anaesthesia, these rats were not exposed to irradiation. The rats in group B were, in addition, given an injection of 10 mg testosterone (Intervet, Johannesburg).

The remaining anaesthetised rats had the lower part of their bodies covered with lead protective wear. The testes were exposed to a 60Co gamma-ray unit (Source S-4089). Single doses of 3.5 or 6.0 Gy were administered at a dose rate of 0.96 Gy/minute.
Rats in groups C and E were subjected to single doses of 3.5 Gy irradiation, whereas those in groups D and F were subjected to 6.0 Gy of irradiation (Figure 2.1).

The rats were injected (i.m.) with 0.25 mg Rompun (Bayer, Isando) immediately after removal from the gamma ray unit. Later the rats were taken to their housing cages and kept between 4 and 8 weeks, depending on the sub group. They were weighed weekly.

At the end of the recovery period, the rats were transported to Onderstepoort where the analyses were performed. The rats were killed by administering an over-dosage of halothane anaesthetic.
2. 2. 2. **METHOD**

**MALE RATS**

GROUP A | GROUP B | GROUP C | GROUP D | GROUP E | GROUP F
---|---|---|---|---|---
No irradiation | No irradiation | 3.5 Gy irradiation | 6.0 Gy irradiation | 3.5 Gy irradiation | 6.0 Gy irradiation
No testosterone | Testosterone | No testosterone | No testosterone | Testosterone | Testosterone

Killed: 4 wk | 8 wks | 4 wks | 8 wks | 4 wks | 8 wks | 4 wks | 8 wks | 4 wks | 8 wks | 4 wks | 8 wks
n=8 | n=8 | n=8 | n=8 | n=8 | n=8 | n=8 | n=8 | n=8 | n=8 | n=8 | n=8

**PROCEDURES**

- **TESTIS REMOVED**
  - Tissue histology and Repopulation index determination- Light microscopy– **CHAPTER 2**

- **EPIDIDYMIS REMOVED**
  - Sperm motility studies – Computer Assisted sperm motion analysis (CASA)- **CHAPTER 3**

- **BLOOD COLLECTED-CARDIAC PUNCTURE**
  - Plasma hormone level determination (LH, FSH, Testosterone) by Radioimmunoassay– **CHAPTER 4**

**FIGURE 2. 1.** Schematic representation of the experimental protocol
2.2.1. TISSUE SAMPLING

Following the collection of blood from the heart, the testes were removed. The left testis was cleaned up and weighed. The testis/body mass index was then calculated from the values obtained.

The testes were cut into 5 mm x 5 mm blocks. The blocks were transferred into 2 ml micro centrifuge tubes containing Bouin’s fluid. The specimens were processed for viewing under the light microscope as described by Hartley (1979). Histological evaluation was performed and the repopulation index calculated.

2.2.2. SPECIMEN PROCESSING

The fixed testes were decolorized in freshly prepared 2% lithium carbonate, and washed in distilled water. The testes were washed several times in acidified water, ammonia water, and rinsed in distilled water to increase detail of nuclear staining. The samples were worked through a graded series of alcohol (5%-100% ethanol) into xylene, and embedded in paraffin. Five µm sections were prepared. The samples were stained in haematoxylin for 2 minutes and then mounted on slides with malinol. Counterstaining was not necessary. Slides were viewed with a Nikon Optiphoto Microscope (Nikon-USA). Photomicrographs were taken with a Nikon camera attached to the microscope.
2.2.3. HISTOLOGICAL EVALUATION OF SPERMATOGENIC ACTIVITY

The repopulation or recovery of the seminiferous epithelium was assessed by determining the repopulation index using an optiphot microscope (Nikon-USA). A 40x objective was used. An average of two hundred tubules per cross section of the slide, representing different sections of the testis, were counted in one testis.

A tubule was scored as repopulating if it contained at least three cells that had reached the type B spermatogonial stage or later. Type A and intermediate spermatogonia were excluded because they are difficult to differentiate from spermatogonial stem cells. Also, M. Meistrich (pers. comm.) suggested that it was operationally more efficient to score these cells as non-repopulating. The percentage of scored tubules was then calculated. This percentage represented the repopulation index.

2.2.3. CALCULATING THE REPOPULATION INDEX (RI)

RI was calculated using the formula:

\[
\text{Number of repopulating tubules} \times 100 \quad \text{(Kangasniemi et al., 1996b)}
\]

\[
\text{total number of counted tubules}
\]
2. 2. 4. DATA AND STATISTICAL ANALYSIS

The General Linear Models (GLM) analysis of variance procedure was used in this analysis (Lepati D, Statistician, MEDUNSA). The Software packages used were SAS for analysis (Lepati D, MEDUNSA), and STATA for verification (Becker P, MRC). Analyses were performed based on three classifications: hormone treatment-2 levels (0; testosterone), irradiation (0; 3.5; 6.0), and weeks (4; 8).

Interaction between irradiation and weeks, hormone treatment and weeks, and irradiation and hormone treatment were tested. The data are represented as arithmetic mean ± SD (standard deviation). The coefficient of variation (CV) and coefficient of determination ($r^2$) were calculated. The coefficient of determination gives the strength of the relationship between irradiation and the testicular index or repopulation index.
2. 3. RESULTS

2. 3. 1. TESTIS/BODY MASS INDEX (TESTICULAR INDEX)

**FIGURE 2. 2.** Histogram representing the testicular index of Sprague Dawley rats following their exposure to irradiation;

4 and 8 = time in weeks after irradiation.

4T and 8T = time in weeks after irradiation in testosterone treated rats.

The word ‘control’ is used to denote the control group that did not receive testosterone.

Figure 2.2 depicts that there was a decrease in testicular index in the testosterone treated control group when compared to the control ($F_{5,1}=9.1; P=0.062$). Four weeks following exposure of the rats to 3.5 Gy of irradiation, the index decreased when compared to the 4 week control ($F_{5,1}=2.83; P=0.003$). There was slight recovery in this 3.5 Gy irradiated group after 8 weeks without testosterone intervention compared to the 3.5 Gy 4 week group ($F_{5,1}=38.49; P=0.06$). However testicular index in this 8 week group was still decreased compared to the 8 week control ($F_{5,1}=8.4; P=0.2$).
There was an increase in the index in the 3.5 Gy 4 week group given testosterone compared to the 3.5 Gy 4 week group that did not receive testosterone ($F_{5.1} = 2.3; P=0.02$). This increase in testicular index was insignificant compared to the testosterone treated 4 week control ($F_{5.1} = 16.5; P=6.72$). Testicular index increased to reach control levels after treatment with testosterone in the 8-week sub-group when compared to the 3.5 Gy 8-week group not administered testosterone ($F_{5.1} = 13.28; P=0.0005$).

Irradiation levels of 6.0 Gy resulted in a pronounced decrease in testicular index after 8 weeks when compared to the control rats killed at the same time ($F_{5.1} = 19.28; P=0.0001$). Testosterone treatment did not result in any significant recovery in the index in these rats when compared to the testosterone treated control rats killed at the same time ($F_{5.1} = 22.8; P=0.239$).
2. 3. 2. HISTOLOGICAL ANALYSIS

A1- Control (no testosterone treatment)
A2- one tubule from the control (magnification x 205)

B1- Testosterone treated control; the arrows are pointing at spermatogonia
B2- Testosterone treated control

FIGURE 2. 3. Photomicrographs (magnification x 76) of sections of rat testes. No visible destruction of spermatogenic cells can be seen. Cells in various stages of spermatogenesis can be seen. S- spermatogonia, Sy- spermatocytes, Sd- spermatids
D2- 6 Gy irradiated testis 8 weeks after treatment with testosterone. Few spermatogenic cells are present. Some of the cells are close to the basal membrane of the tubule; a few are slightly further away. R indicates a repopulating tubule.

C1- 3.5 Gy irradiated testes after 8 weeks; Few cells can be seen.

C2 - 3.5 Gy irradiated testes after 8 weeks after testosterone treatment. The tubules show repopulation.

D1- 6 Gy irradiated testis after 8 weeks with no testosterone treatment. No spermatogenic cells are observable.

D2- 6 Gy irradiated testis 8 weeks after treatment with testosterone. Few spermatogenic cells are present. Some of the cells are close to the basal membrane of the tubule; a few are slightly further away. R indicates a repopulating tubule.

**FIGURE 2.4.** Photomicrographs (magnification x 76) of rat testes exposed to irradiation.
2.3.3. REPOPULATION INDEX (RI)

TABLE 2.1. Repopulation indices 4 and 8 weeks after irradiation with 0, 3.5 and 6.0 Gy γ-irradiation.

<table>
<thead>
<tr>
<th>TIME (WEEKS)</th>
<th>IRRAD (Gy)</th>
<th>TESTOSTERONE</th>
<th>RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>None</td>
<td>100 ± 0.97</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>T</td>
<td>100 ± 0.71</td>
</tr>
<tr>
<td>4</td>
<td>3.5</td>
<td>None</td>
<td>27.3 ± 3.70</td>
</tr>
<tr>
<td>4</td>
<td>3.5</td>
<td>T</td>
<td>65.6 ± 3.82</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>None</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>T</td>
<td>13.8 ± 3.06</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>None</td>
<td>100 ± 0.77</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>T</td>
<td>100 ± 0.80</td>
</tr>
<tr>
<td>8</td>
<td>3.5</td>
<td>None</td>
<td>33.5 ± 3.29</td>
</tr>
<tr>
<td>8</td>
<td>3.5</td>
<td>T</td>
<td>83.7 ± 3.01</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>None</td>
<td>0 ± 0.17</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>T</td>
<td>16.5 ± 1.29</td>
</tr>
</tbody>
</table>

n = 8 ; RI = MEAN ± SD; CV = 5.280; P< 0.05; r² = 0.980

There was a slight decrease in the repopulation index (RI) in the 3.5 Gy irradiated group 4 weeks after exposure compared to the control (F₆₋₂=8.94; P= 0.0037). The RI improved slightly 8 weeks after irradiation compared to the control (F₆₋₂=4.24; P=0.06). There was an increase in the repopulation index of these irradiated rats 4 weeks following treatment with testosterone compared to the testosterone treated control (F₆₋₂=4.19; P=0.0012). The increase was more pronounced following 8 weeks of testosterone treatment (F₆₋₂=3.57; P=0.0005). The results show no repopulation for the 6.0 Gy dose group over 4 and 8 weeks without testosterone treatment.
2.4. DISCUSSION

2.4.1. TESTIS/BODY MASS (TESTICULAR) INDEX

There was no statistically significant decrease in testisicular index in the hormone treated control compared to the non-treated control. Testosterone administration therefore did not affect testicular index. Irradiation of the rat testes resulted in a statistically significant dose dependant decrease in testicular index.

It is noteworthy that following exposure of the rats to irradiation, their mean body weight increased throughout the experiment and was not different from the mean body weight of the age-matched controls. Therefore the decrease in testicular index may be attributed to a reduction in testis mass. Similarly, studies conducted by Wang et al. (1993) involving exposure of Sprague Dawley rats to 5.27 Gy of gamma irradiation resulted in a fall in testis weight. Cunningham and Huckins (1978), likewise, reported a gradual loss in testis weight following exposure of the rat testis to low dose gamma irradiation. Interestingly, Pinon-Lattaillade et al. (1991) reported a decline in the testis weight of rats subjected to a dose as high as 9.0 Gy gamma irradiation compared to non-irradiated controls. No significant recovery of testis weight was observed even at 180 days following irradiation exposure (Pinon-Lattaillade et al., 1991).

Following testosterone administration, testicular index in the 3.5 Gy dose group increased to a level comparable to that of the control. This is in agreement with the findings of Wilson et al. (1999) who treated rats with testosterone plus oestradiol prior to exposing them to 3.5 Gy gamma irradiation.
The testosterone plus oestradiol treated rats showed a significantly higher testis weight when compared to irradiated cholesterol-treated controls (Wilson et al., 1999). The administration of testosterone to rats following their exposure to irradiation may have lead to a recovery in testicular weight.

2.4.2. MORPHOLOGICAL CHANGES

All tubules in the control exhibited ‘active’ spermatogenesis since different cell populations at various stages of spermatogenesis were present.

In the 3.5 Gy dose group of rats without hormone treatment, there were fewer spermatogenic cells. This may have been a result of destruction of the germ cells by irradiation. Previous studies on the effects of low dose gamma irradiation (in particular 3.0 - 3.5 Gy) have reported a decrease in spermatogenic cell numbers (Meistrich and Kangasniemi, 1997). Cunningham and Huckins (1978) found that only 11% of spermatogonia survived after 3.0 Gy irradiation. In contrast, in the group treated with testosterone, the majority of tubules were repopulated with spermatogenic cells at various stages. This is strongly indicative of spermatogenic recovery.

In the 6.0 Gy dose group, the majority of tubules contained virtually no spermatogenic cells. Erickson(1976) reported a 99% reduction in A1 spermatogonial cell numbers following exposure of Sprague Dawley rats to 6.0 Gy γ-irradiation. In this study, only a few cells situated close to the basement membrane of the seminiferous tubules were seen. These cells may have been spermatogonial stem cells. These stem cells have been shown to be the most resistant cells to irradiation. Studies investigating the effect of 6.0 Gy irradiation have shown that at this dose cell depletion takes place and that no recovery of spermatogenesis occurs even 20 weeks following irradiation.
However after testosterone administration, tubules containing small colonies of spermatogenic cells with a few type B cells, were observed. It is difficult to deduce from this observation whether there was recovery. Quantitative morphological techniques, like repopulation index determination, would be useful in this regard.

2. 4. 3. REPOPULATION INDEX

The repopulation indices showed that in groups A and B (controls), the repopulation index (RI) was 100 %. No damage was induced by irradiation on the germ cells. In group C (rats exposed to 3.5 Gy irradiation and not treated with testosterone), the repopulation index decreased by 73% when compared to the control (group A). This massive decrease in RI was observed despite the presence of surviving spermatogonial cells (photo C1, histology results). A similar observation has been reported in previous studies on the effects of low dose $\gamma$-irradiation. However the inability of these surviving spermatogonial cells to repopulate the seminiferous epithelium remains an enigma.

In the testosterone treated rats (group E), the RI increased to 84%. Wilson et al. (1999) observed an increase in RI to some 99% of the control value in rats pre-treated with exogenous testosterone and oestradiol and finally exposed to 3.5 Gy irradiation.

In the 6.0 Gy dose group, the RI was 0 % at both 4 and 8 weeks. The 6.0 Gy non- testosterone treated group (D) showed no recovery of spermatogenesis while rats treated with testosterone exhibited a RI increase from 0 % to 16.4 %. This slight recovery in spermatogenesis occurred possibly because of the severity of testicular damage at this dose. It is interesting to note that Meistrich and Kangasniemi (1997) exposed rats to 6.0 Gy $\gamma$-irradiation and treated them with Zoladex.
CHAPTER 3. EFFECTS OF GAMMA IRRADIATION ON CAUDA EPIDIDYMAL SPERM MOTILITY IN SPRAGUE DAWLEY RATS

3.1.1. INTRODUCTION

Motility is one of the most important fundamental parameters that determine the physiological state of the sperm (Samuels and van der Horst, 1986). The analyses of motility characteristics in a number of rat species have revealed significant correlations between various motion characteristics and sperm function (Samuels and van der Horst 1986; Kaskar et al., 1994).

Overstreet et al. (1998) investigated the effects of several toxicants on various parameters with the aim of identifying early indicators of toxicity to the testes. A consistent finding of these studies was that changes in sperm output occurred concomitantly with changes in sperm motility. From this, Overstreet et al. (1988) concluded that sperm motility was an invaluable tool for investigating testicular toxicity. Furthermore, van der Horst et al. (1999) described changes in motility as being conspicuous, thus, sperm kinematics may provide a good indication of the damage caused by low dose irradiation to the testes.

3.1.2. EFFECT OF IRRADIATION ON SPERM MOTILITY

Several researchers have studied the effects of irradiation on sperm motility in several rat strains. A review of these studies was compiled to give a background on how sperm motility is affected by irradiation.
Bansal et al. (1990) investigated the effects of 2.5 and 10.0 Gy $\gamma$-irradiation on spermatogenesis and epididymal sperm in albino rats. Irradiation was found to cause a decrease in the number of epididymal sperm after only 4 weeks and caused a decrease in the proportion of non-motile sperm. These effects of irradiation on epididymal sperm were increased with an enhanced dose (Bansal et al., 1990).

Okamoto (1992) exposed hamsters to doses of between 0.25-6.0 Gy X-ray irradiation. Spermatozoa were collected from the cauda epididymis at different time intervals (2-6 weeks) following irradiation, and the motility was subsequently analysed. A decrease in both sperm concentration and sperm motility was found following exposure of the testes to irradiation doses above 0.5 Gy (Okamoto, 1992).

A similar study used to evaluate the effect of low dose gamma irradiation on epididymal sperm in adult rats was conducted by Varga et al. (1994). Doses below 2.0 Gy of $\gamma$-irradiation did not produce any significant effect on epididymal sperm in terms of the numbers or percentage motility. Whereas exposure to irradiation above 3.0 Gy resulted in a decrease in the number of epididymal sperm and likewise, in the percentage of motile sperm.
3.2. MATERIALS AND METHOD

After each rat was killed, the testes were removed. Testes were transferred into a 58 x 15 mm petri dish containing 4 ml nutrient mixture Ham’s F-10 medium (Sigma-St Louis) pre-warmed to 36 °C. Adipose tissue, connective tissue, and blood vessels were dissected from the right testis and epididymis.

The epididymis was separated from the associated testis and transferred into a 50 x 9 mm petri dish containing fresh Ham’s F-10 medium. A small incision was made in the caudal area of the epididymis. Sperm were aspirated from the cauda epididymis and transferred into a 35 mm petri dish with fresh warm medium. A volume of 7 µl of the suspension was transferred onto a warm microscope glass slide and covered with a 20 mm x 20 mm cover slip. The sperm were then viewed under a light microscope using negative phase contrast optics and fitted with a slide warmer and a digital camera attached to a VHS video machine to allow for the recording of motile sperm. Video images of motile sperm were analyzed by means of computer assisted sperm analysis using the sperm motility quantifier (SMQ, Wirsam Scientific and Precision Equipment- Johannesburg). The recordings of sperm motion were captured at an analysis rate of 50 Hz. An average of 100 motile spermatozoa were analyzed per animal.

Percentage (sperm) motility was determined by calculating the number of motile sperm/ total number of sperm per field x100. Five kinematic parameters were measured: VCL (Curvilinear velocity), VAP (average path velocity), VSL (Straight line velocity), mnALH (minimum amplitude of lateral head displacement) and mxALH (Maximum amplitude of lateral head displacement).
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>UNIT</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curvilinear velocity (VCL)</td>
<td>µm/sec</td>
<td>Time-average velocity of sperm head along its actual path</td>
</tr>
<tr>
<td>Straight line velocity (VSL)</td>
<td>µm/sec</td>
<td>Straight line between its first and last detected position</td>
</tr>
<tr>
<td>Average path velocity (VAP)</td>
<td>µm/sec</td>
<td>Time-average velocity of sperm head projected Along its spatial average trajectory</td>
</tr>
<tr>
<td>Maximum amplitude of lateral</td>
<td>µm/sec</td>
<td>Maximum amplitude of lateral distances of the sperm head trajectory about its spatial average path.</td>
</tr>
<tr>
<td>head displacement (mxALH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum amplitude of lateral</td>
<td>µm/sec</td>
<td>Maximum amplitude of lateral distances of the sperm head trajectory about its spatial average path.</td>
</tr>
<tr>
<td>head displacement (mnALH)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3. STATISTICAL ANALYSIS

Data analysis involved the General linear model (GLM) analysis of variance procedure. This model was used to determine whether there was a relationship between irradiation and motion parameters, and to determine if there is a relationship between exogenous testosterone and motion characteristics. It is one of the popular models employed for parametric analysis in medical and biological studies (Dawson-Saunders and Trapp, 1994). The coefficient of determination ($r^2$) between irradiation and each motility parameter analyzed in this chapter was calculated - (Statisticians: Becker P, Medical Research Council; Lepati D, Medunsa).
3. 4. RESULTS

3. 4. 1. PERCENTAGE MOTILITY

% Sperm motility was calculated as mean ± SD; n = 8; P < 0.05; $r^2 = 0.727$

**FIGURE 3.1:** Histogram showing percentage sperm motility

There was no difference in the percentage sperm motility between the testosterone-treated and non-treated rats killed after 4 weeks ($F_{6,1}=3.1; P=1.62$) or in rats killed after 8 weeks ($F_{6,1}=5.2; P=0.75$). Irradiation caused a decrease in sperm motility at 4 weeks post exposure compared to the control ($F_{6,1}=4.7; P=0.03$). No increase in motility was observed in testosterone treated rats killed after 4 weeks ($F_{6,1}=4.5; P=0.14$) or 8 weeks compared to testosterone treated controls ($F_{6,1}=0.11; P=0.73$).

**C-4** and **C-8**:- Control rats killed after 4 weeks and 8 weeks

**C-4 T** and **C-8 T**:- Testosterone-treated control rats killed after 4 and 8 weeks

**3.5 Gy-4** and **3.5 Gy-8**:- Rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation

**3.5 Gy-4 T** and **3.5 Gy-8 T**:- Testosterone treated rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
3.4.2. KINEMATIC PARAMETERS

FIGURE 3.2: Histogram showing curvilinear motion (VCL)

There was no change in VCL in the hormone treated control killed after 4 weeks when compared to the non-treated control killed at the same time (F_{6,1}=7.1; P=0.41). Exposure of the rat testis to 3.5 Gy of gamma irradiation resulted in a decrease in VCL (F_{6,1}=0.9; P=0.001). VCL remained significantly decreased 8 weeks after irradiation compared to control rats killed after 8 weeks (F_{6,1}=6.7; P=0.2). Testosterone treatment did not result in any significant changes in VCL compared to the testosterone treated control (F_{6,1}=5.2; P=0.37). However, there was no change in VCL in testosterone treated rats killed after 4 weeks.

C-4 and C-8:- Control rats killed after 4 weeks and 8 weeks
C-4T and C-8T:- Testosterone-treated control rats killed after 4 and 8 weeks
3.5 Gy-4 and 3.5 Gy-8:- Rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
3.5 Gy-4T and 3.5 Gy-8T:- Testosterone treated rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation

VCL = mean ± SD. n = 8; P < 0.05; r^2 = 0.721
STRAIGHT LINE VELOCITY (VSL)

VSL was calculated as the mean ± SD. n = 8; P < 0.05; r² = 0.659

**FIGURE 3.3:** Histogram showing straight line velocity (VSL)

VSL was the same in testosterone-treated control rats and non-treated control rats killed after 4 weeks (F₀₁=4.5; P=0.2). However, VSL was slightly increased in the 4 week testosterone treated control compared to the 8 week testosterone-treated control (F₀₁=5.7; P=0.26). There was a significant decrease in VSL following 3.5 Gy gamma irradiation of the rat testis compared to the non-testosterone treated control (F₀₁= 5.2; P=0.03). Testosterone treated rats in this dose group showed an increase in VSL when compared to the control rats sacrificed at the same time (F₀₁=3.1; P=0.04). However, VSL decreased in this testosterone-treated group after 8 weeks when compared to the 4 week group (F₀₁= 8.1; P = 0.04).

C-4 and C-8:- Control rats killed after 4 weeks and 8 weeks
C-4T and C-8T:- Testosterone-treated control rats killed after 4 and 8 weeks
3.5 Gy-4 and 3.5 Gy-8:- Rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
3.5 Gy-4T and 3.5 Gy-8T:- Testosterone treated rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
VAP was calculated as the mean ± SD. n = 8; P < 0.05; r² = 0.622

**FIGURE 3.4:** Histogram showing average path velocity (VAP)

There was no significant change in VAP in the control following testosterone treatment compared to non-treated control rats killed after 4 weeks (F₆,₁=5.2; P=0.3)

A dose of 3.5 Gy of gamma irradiation resulted in a decrease in VAP when compared to the control (F₆,₁=1.4; P=0.04). VAP remained decreased 8 weeks after the exposure of the rats to irradiation when compared to the control rats killed after 8 weeks (F₆,₁=6.7; P=0.31). VAP was decreased in testosterone treated rats sacrificed after 8 weeks compared to testosterone-treated rats sacrificed after 4 weeks (F₆,₁=7.3; P=0.63).

**C-4 and C-8:** Control rats killed after 4 weeks and 8 weeks

**C-4T and C-8T:** Testosterone-treated control rats killed after 4 and 8 weeks

**3.5 Gy-4 and 3.5 Gy-8:** Rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation

**3.5 Gy-4T and 3.5 Gy-8T:** Testosterone treated rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
FIGURE 3.5: Histogram of minimum amplitude of lateral head displacement (mnALH). There was no change in mnALH in testosterone-treated control rats killed after 4 weeks ($F_{6,1} = 3.2; P=1.7$) and rats killed after 8 ($F_{6,1} = 5.1; P=2.8$) weeks compared to non-treated control rats killed at the same time. Irradiation resulted in an that was statistically not significant decrease in mnALH ($F_{6,1} =0.6; P=0.26$). Based on correlation studies between time after treatment (4 and 8 weeks), and irradiation, the time of exposure did not make a difference in mnALH ($F_{6,1} =2.6; P=0.41$). Hormone treatment did not lead to any improvement in mnALH in rats killed after 4 weeks compared to testosterone-treated control rats killed at the same time ($F_{6,1} = 4.2; P=0.58$).

**C-4 and C-8**: Control rats killed after 4 weeks and 8 weeks

**C-4T and C-8T**: Testosterone-treated control rats killed after 4 and 8 weeks

**3.5 Gy-4 and 3.5 Gy-8**: Rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation

**3.5 Gy-4T and 3.5 Gy-8T**: Testosterone treated rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
FIGURE 3.6: Histogram showing maximum amplitude of lateral head displacement (mxALH). There was an increase in mxALH in testosterone-treated control rats killed after 4 weeks compared to non-treated control rats killed at the same time ($F_{6,1}=1.62; P=0.15$). An increase in mxALH was also observed in testosterone-treated control rats killed after 8 weeks compared to non-treated rats killed at the same time. However, mxALH was decreased in the testosterone-treated control rats killed after 8 weeks compared to those of 4 weeks ($F_{6,1}=5.3; P=0.19$). Exposure of the rats to 3.5 Gy gamma irradiation resulted in a slight decrease in mxALH at 4 weeks ($F_{6,1}=0.7; P=0.75$) compared to the control (group A). Testosterone treatment did not alter mxALH in rats killed after 4 weeks ($F_{6,1}=4.1; P=0.09$) and 8 weeks ($F_{6,1}=3.2; P=0.12$) when compared to testosterone-treated control rats killed after 4 and 8 weeks respectively.

C-4 and C-8:- Control rats killed after 4 weeks and 8 weeks
C-4T and C-8T:- Testosterone-treated control rats killed after 4 and 8 weeks
3.5 Gy-4 and 3.5 Gy-8:- Rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
3.5 Gy-4T and 3.5 Gy-8T:- Testosterone treated rats killed 4 and 8 weeks after exposure to 3.5 Gy irradiation
No results are reported for the 6.0 Gy dose group. No motile sperm were observed, all sperm being decapitated. These observations were consistent both 4 and 8 weeks following irradiation exposure. Testosterone administration did not result in any observable changes.
3. 5. DISCUSSION

3. 5.1. SPERM MOTILITY QUANTIFICATION

The percentage sperm motility did not differ in the testosterone treated and non-treated controls. Interestingly, Wilson et al. (1999) reported that exogenous testosterone results in a decline in sperm numbers in rats. Although it is possible that sperm numbers can be affected following the administration of exogenous testosterone, percentage sperm motility is not altered.

In rats exposed to a dose of 3.5 Gy the percentage sperm motility declined 4 weeks post irradiation. No further decrease in percentage motility was observed 8 weeks subsequent to exposure to irradiation. Bansal et al. (1990) similarly reported a reduction in percentage sperm motility 4 weeks following exposure of albino rats to 2.5 Gy γ-irradiation. Interestingly, no further change was observed in percentage sperm motility 16 weeks following exposure to irradiation. The effects of γ-irradiation on epididymal sperm observed in this study may have been due to direct physical damage. This observation is supported by results following 6.0 Gy irradiation, where cells were decapitated. Abnormalities in sperm tail morphology were revealed in the study conducted by Overstreet et al. (1988).

Epididymal sperm may be susceptible to radiation damage due to decreased androgen binding protein (ABP) levels. The maturation of sperm in the epididymis depends on the availability of sufficient quantities of testosterone. Testosterone is transported to the epididymis bound to ABP, which has been shown to decrease following exposure of the testis to radiation.
Galdieri et al. (1984) showed that there was a distinct relationship between late spermatids and ABP levels, a decrease in the number of late spermatids being closely associated with a decline in ABP concentration (Galdieri et al., 1984).

Radiation led to an elimination of spermatogenic cells (including spermatids). Pineau et al. (1989) found that ABP levels returned to normal with the recovery of spermatogenesis following irradiation.

Percentage motility did not improve following the administration of testosterone over the periods 4 and 8 weeks. Hence testosterone administration does not lead to enhanced sperm motility in rats exposed to 3.5 Gy $\gamma$-irradiation.
3.5.2. KINEMATIC PARAMETERS

Of the parameters analyzed, VCL and VSL were significantly lowered following exposure of the rat testis to 3.5 Gy of irradiation when compared to controls not treated with testosterone. VCL and VSL are two of the three measures of sperm vigor. Overstreet et al. (1988) exposed beagle dogs to whole body gamma irradiation and observed decreased sperm swimming velocity. VAP, the other parameter that gives a measure of sperm vigor, was unaffected. No recovery or further decline in the magnitude of the aforementioned parameters was observed after 8 weeks, further supporting the possibility of a physical effect of irradiation on epididymal sperm.

Testosterone administration did not result in any improvement in VCL. In contrast VSL, which had decreased following exposure to 3.5 Gy irradiation, recovered towards the control values 4 weeks following testosterone treatment. The VSL values were similar to those of testosterone non-treated 3.5 Gy exposed rats after 8 weeks. The seemingly improved VSL values at 4 weeks may have been transient, but the finding cannot be ignored since the improvement was significant.

Lateral oscillation parameters, mxALH and mnALH showed statistically insignificant fluctuations following irradiation of the testis in the 3.5 Gy dose group. Testosterone treatment did not affect the two parameters. These parameters may be unaffected by exposure to low dose gamma irradiation. Also, they may lack susceptibility to exogenous testosterone at the administered dose. Therefore VCL and VSL, which were consistently and significantly affected by low dose gamma irradiation, could be used to monitor testicular damage induced by γ-radiation.
CHAPTER 4: EFFECTS OF IRRADIATION ON THE ENDOCRINE PROFILES OF SPRAGUE DAWLEY RATS

4.1. INTRODUCTION

Communication between the principal testicular cells (Leydig, Sertoli, and germ cells) plays a crucial role in the regulation of spermatogenesis in mammals (Sharp and Fraser, 1980; Sharp, 1986; Jegou, 1993). The control of testicular function also involves a cocktail of hormones (Yen and Jaffe, 1978). Research has shown that there is an intricate interplay between the above-mentioned testicular cells and reproductive hormones in regulating testicular function (Jegou, 1993). In chapter 2 it was shown that exposure of the testes to low dose gamma irradiation results in germ cell loss. A critical view of the effects of germ cell loss on the hormonal control of testicular function is given later in the chapter.

4.1.1. HORMONAL CONTROL OF TESTICULAR FUNCTION

The major hormones involved in the regulation of spermatogenesis are: gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), Luteinising hormone (LH), androgens (mainly testosterone), and inhibin. The hormonal regulation of spermatogenesis involves intricate interactions between these hormones (the pathways of this regulatory process are represented in figure 4 below).
FIGURE 4.1: Schematic diagram depicting the hypothalamic-pituitary-gonadal axis showing some of the major hormones regulating spermatogenesis (Yen and Jaffe, 1978). Dotted lines indicate negative feedback pathways. FSH is required for the initiation of spermatogenesis. Testosterone is required in the later stages (the division of primary spermatocytes, DHT is required for the differentiation of spermatids.
4.1.2. HORMONAL CHANGES FOLLOWING IRRADIATION OF THE TESTES

Extensive studies on the endocrinological changes following acute irradiation of the rat testis have been undertaken (Galdieri et al., 1984; Pineau et al., 1989). Low dose irradiation results in an increase in plasma FSH concentration (Pinon-Lataillade et al., 1985; Pineau et al., 1989; Kudorglu et al., 1994; Kangasniemi et al., 1996a; Meistrich et al., 1996). These studies have further shown that plasma LH concentrations rise, though to a lesser degree than FSH (Meistrich and Kangasniemi, 1997). In marked contrast, Cunningham and Huckins (1978) found no significant changes in the levels of plasma FSH and LH following irradiation.

Plasma testosterone levels undergo slight insignificant changes (Meistrich and Kangasniemi, 1997). Cunningham and Huckins (1978) did not observe any changes in plasma testosterone following exposure of Sprague Dawley rats to low-level $\gamma$-irradiation. The levels of androgen binding protein (ABP), which binds testosterone and avails it to the dividing spermatogenic cells, has been shown to decrease following exposure of the testis to low dose irradiation (Pineau et al., 1989).

While plasma testosterone concentrations have been shown to be unaffected by low dose $\gamma$-irradiation, the levels of intratesticular testosterone (ITT) increase 200 fold (Pinon-Lataillade et al., 1985; Pineau et al., 1989; Wang et al. 1993; Kudorglu et al., 1994; Kangasniemi et al., 1996a; Meistrich et al., 1996). The 200 fold increase in ITT is compounded by a profound reduction in ABP, which ‘buffers’ testosterone levels in the testes (Wang et al., 1993).
The explanation put forward by Wang et al. (1993) for high ITT and unaffected plasma testosterone levels, is the impairment of testicular blood flow due to damage of the testes following irradiation.

In this chapter the effects of irradiation on the hormone profile of Sprague Dawley rats were investigated. The plasma concentrations of LH, FSH, and testosterone prior to and following exposure to low levels of gamma irradiation were measured. In addition, the influence of exogenous testosterone on the plasma profiles of these hormones was determined.
4. 2. MATERIALS AND METHODS

4. 2. 1. BLOOD SAMPLE COLLECTION AND PREPARATION

Rats were overdosed with halothane (anaesthetic procedures were all performed under the care and supervision of a veterinarian), after which the chest cavity was opened. Blood samples were collected by cardiac puncture using 10-ml sterile K₂EDTA coated vacutainer tubes connected to a hypodermic needle. The samples were centrifuged at 300x g for 15 minutes. Following centrifugation the plasma was transferred to 1.5 ml eppendorf tubes and stored at -20 °C for three months prior to assaying. The plasma was collected for the determination of LH, FSH, and testosterone concentrations.

4. 2. 2. RADIO-IMMUNOASSAY OF LH AND FSH

LH and FSH were assayed using the double antibody radioimmunoassay (RIA) method as described by Clayton et al. (1980). Assays for LH and FSH were performed individually, however the same procedure was followed for the assay of each hormone, except different antibodies and reagents were used. The term hormone has thus been used indiscriminately to denote either LH or FSH. NIDDK rLH RP-2 and rFSH RP-2 were used as standards for LH and FSH assays respectively. An amount of 50 µl of rat plasma diluted 1:100 with phosphate buffer (pH 7) was used.

4. 2. 2. 1. ASSAY VALIDATION:

Validation is the presentation of documented evidence that all causes for variation have been accounted for, and that any variation present will not be excessive of expected variation or standard curve variation (Midgeley et al., 1969).
Analytical variables for the validation of RIA include Specificity, Sensitivity, and Reproducibility (Blake et al., 1973).

a). Specificity

The specificity of an assay can be defined as the degree to which an assay responds to substances other than that for which it was designed (Chard, 1990). In the case of LH and FSH, to ensure specificity and minimise cross reactivity, antisera to the β sub-unit was used.

LH and FSH consist of 2 sub-units, the α and β sub-units. The α sub-unit is identical in the four adenohypophysis glycoprotein hormones, FSH, LH, ACTH (adenocorticotropic hormone), and TSH (thyroid stimulating hormone). The β sub-units are different for each hormone and confers biological specificity on each of these hormones (Yen and Jaffe, 1978).

To test for assay specificity, a test for parallelism was performed for each hormone. Serial dilutions of a plasma sample paralleled a corresponding standard curve (figure 4.2).
FIGURE 4.2. Semilogarithmic plot of: A- rat LH standards (I) and serial dilutions of plasma (II); B- rat FSH standards (III) and serial dilutions of plasma (IV). The degree of parallelism that exists between the dose-response curves of rat plasma and hormone standards suggests little or no cross-reactivity with other plasma borne hormones.

b). Sensitivity

Sensitivity, defined as the lowest level of hormone detected and twice the standard deviation of the buffered blank was 0.08 ng/ml for LH and 5.2 ng/ml for FSH.
c). Reproducibility

Reproducibility, also referred to as precision, is a measure of the variation observed between repeated determinations on the same sample. To determine reproducibility, samples were assayed in triplicate; the mean, standard deviation (SD), and CV were determined. The intra-assay CV (within a run) for LH was 10.3 % and for FSH was 11.0 %. The inter-assay CV (between runs) for LH was 15.3 % and was 16.7 % for FSH.

4.2.3. RADIO-IMMUNOASSAY OF TESTOSTERONE

Testosterone concentrations were measured by radioimmunoassay. The assay was performed according to the manufacturer’s instructions. A Coat-A-Count kit (Diagnostic corporation, USA) was used as per manufacturer’s instructions. Coat-A-Count-A-Count is a no extraction, solid phase radioimmunoassay and it is strictly for in vitro diagnostic use. For testosterone assay 50 µl of plasma was used.

The radioimmunoassay was validated for use in the Sprague Dawley rats by serially diluting the plasma (over the range 1:1 to 1:32) and checking that the displacement curve of a series of double dilutions paralleled the standard curve. The regression of sample and standard curves were not significantly different (ANOVA) $F = 0.11; P = 0.75$. The antibody is highly specific for testosterone. Cross reactivity with all naturally occurring steroids is $> 0.05 \%$, except for 5-$\alpha$ dihydrotestosterone for which it is 3%. The sensitivity of the assay is 0.14 nmol/l. The intra-assay coefficient of variation was 6.4 %. No inter-assay coefficient of variation is reported as only one assay was undertaken.
4.3. STATISTICAL ANALYSIS

The General Linear Models (GLM) procedure analysis of variance was used to analyse data. Analysis was performed on non-transformed data. The model is used to determine a relationship between two variables. It is one of the popular analytical tools in biological research (Saunders and Trapp, 1994). Irradiated rats were compared to non-irradiated controls to determine whether irradiation has an effect on the reproductive hormone profile following irradiation of the testes. To determine whether hormone treatment can reverse the effects of irradiation, irradiated testosterone treated rats were compared to the testosterone-treated controls. The coefficient of variation was calculated in each case and $r^2$, which gives a degree of the relationship between variables (irradiation and hormone concentration in this chapter), was determined.
LH concentration values are presented as mean ± SD; n = 8; CV = 4.79; P< 0.05; $r^2 = 0.81$

$4$ and $8 = 4$ and $8$ weeks after irradiation; $4T$ and $8T = 4$ weeks and $8$ weeks following treatment with testosterone respectively.

**FIGURE 4. 2:** Histogram representing the mean concentration of LH following exposure of rats to irradiation. LH concentrations were slightly higher in the non-treated control group sacrificed at $8$ weeks compared to rats in the same group sacrificed at $4$ weeks ($F_{9.2}= 0.12; P=0.31$). No significant difference was observed in the testosterone-treated control group killed at week $4$ compared to the rats in the same group killed at week $8$ ($F_{9.2}= 0.1; P=0.71$). There was an insignificant decrease in LH concentration in the testosterone-treated group when compared to the non-testosterone-treated control at $4$ weeks ($F_{9.2}= 3.14; P=0.1$). A further decrease in plasma LH occurred in control rats given testosterone and killed after $8$ weeks when compared to the non-testosterone-treated control killed at the same time ($F_{9.2}= 0.06; P= 0.09$).
There was an increase in plasma LH concentration 4 weeks following exposure of the rats to an irradiation dose of 3.5 Gy when compared to the control (F$_{9,2}$= 0.01; P= 0.911). A more pronounced increase in LH levels was seen 8 weeks after irradiation (F$_{9,2}$= 3.4; P = 0.03). LH levels decreased slightly after testosterone administration (F$_{9,2}$=0.01; P=0.99). There was a further decrease in LH concentration in the rats treated with testosterone and then sacrificed at 8 weeks compared to the non-testosterone-treated rats in the same group (F$_{9,2}$=3.1; P=0.03). However, when compared to the testosterone treated rats sacrificed at 4 weeks, the increase observed in the 8-week group was not significant (F$_{9,2}$=2.4; P=0.11)

Plasma LH concentration increased significantly 8 weeks after exposure of rat testes to 6.0 Gy $\gamma$-irradiation (F$_{9,2}$=5.64; P=0.002). LH concentration in this dose group decreased following testosterone administration (F$_{9,2}$=3.77; P=0.003).

The levels of LH were unaltered in the 6.0 Gy groups injected with testosterone and sacrificed after 4 weeks compared to rats sacrificed after 8 weeks (F$_{9,2}$=0.25; P=0.82).
FSH levels are presented as mean ± SD, n = 8; CV = 3.02; P < 0.05; r² = 0.85
4 and 8 weeks after irradiation; 4T and 8T = 4 weeks and 8 weeks following treatment with testosterone, respectively

**FIGURE 4.3**: Histogram representing the levels of FSH following exposure to irradiation. No changes in plasma FSH were observed in the 4-week testosterone non-treated control rats compared to rats in the same group killed after 8 weeks (F₀₂=0.6; P=0.53). Plasma FSH showed no significant change in the 4-week testosterone treated control (F₀₂=0.4; P=0.37) compared with the 8-week testosterone treated control. The level of FSH was not affected following testosterone administration in the control rats killed after 4 weeks (F₀₂=3.3; P=0.52) and 8 weeks (F₀₂=4.1; P=0.47) when compared to the non-treated control sacrificed after 4 and 8 weeks respectively.

There was an increase in FSH levels 4 weeks following exposure of the rats to 3.5 Gy γ-irradiation (F₀₂=1.97; P=0.10) compared to the control rats killed after 4 weeks. LH levels rose further 8 weeks following irradiation when compared to the control (F₀₂=0.7; P=0.09).
The rise in FSH in rats killed after 8 weeks was slight when compared to the FSH levels of rats in the same group killed after 4 weeks (F<sub>9.2</sub>=0.26; P=0.93). FSH concentrations were increased in the 3.5 Gy testosterone treated group after 4 weeks compared to the testosterone treated control (F<sub>9.2</sub>=0.57; P=0.13) compared to the testosterone treated control. There was a further increase in FSH after 8 weeks in the testosterone treated group compared to the testosterone treated control sacrificed at the same time (F<sub>9.2</sub>=0.53; P=0.047). No significant difference was seen in LH values in testosterone treated rats killed 4 weeks after irradiation and FSH levels in rats killed 8 weeks following irradiation (F<sub>9.2</sub>=0.64; P=0.87).

There was a more pronounced and significant increase in FSH 4 weeks following exposure to 6.0 Gy irradiation (F<sub>9.2</sub>=3.9; P= 0.003). FSH levels were high 8 weeks following irradiation when compared to the control rats killed at the same time (F<sub>9.2</sub>=4.14; P=0.001). Following testosterone treatment, plasma LH concentrations remained high in rats killed after 4 weeks compared to the testosterone treated control rats killed after 4 weeks (F<sub>9.2</sub>=0.72; P=0.04). Rats in this dose group given testosterone and sacrificed after 8 weeks also showed higher levels of LH compared to the control rats given testosterone and sacrificed after 8 weeks (F<sub>9.2</sub>=0.65; P=0.057). FSH levels did not change significantly over time after testosterone administration, as reflected by compared results of rats sacrificed after 4 weeks and those sacrificed after 8 weeks (F<sub>9.2</sub>=0.76; P=0.473).
Testosterone levels are presented as mean ± SD, n=6, P< 0.05, CV = 6.972, r² = 0.64
4 and 8 = 4 and 8 weeks after irradiation; 4T and 8T = 4 weeks and 8 weeks
following treatment with testosterone, respectively

**FIGURE 4.** Histogram representing the mean concentration of testosterone following the exposure of rats to irradiation. Testosterone levels were slightly lower in the control group not administered testosterone and sacrificed at 8 weeks compared to rats in the same group sacrificed at 4 weeks (F_{6,1}= 2.3; P=0.25). No significant difference in plasma testosterone was observed in the testosterone treated control group killed after 4 weeks compared to the rats in the same group killed after 8 weeks (F_{6,1}=3.5; P=0.52). Plasma testosterone concentrations increased in the testosterone-treated control group killed after 4 weeks compared to the non-testosterone-treated control killed at the same time (F_{6,1}= 3.63; P= 0.02).

Plasma testosterone concentrations increased further in control rats given testosterone and killed after 8 weeks when compared to the non-testosterone treated control killed at the same time (F_{6,1}= 2.5; P= 0.1).

Testosterone levels increased 4 (F_{6,1}=3.5; P=0.08) and 8 weeks (F_{6,1}=3.1; P=0.04) following irradiation in the testosterone treated group when compared to controls sacrificed at the same times.
There was no significant change in the testosterone treated rats killed after 8 weeks when compared to rats in the same group killed after 4 weeks (F\textsubscript{6,1}=3.2; P=0.43).

A decrease in testosterone levels, which was not statistically significant was, observed in the 6.0 Gy dose group 4 weeks after exposure to irradiation when compared to the control at the same time (F\textsubscript{6,1}= 4.81; P=0.08). There was a slight increase in plasma testosterone 8 weeks following exposure to 6.0 Gy irradiation compared to the control group killed after 8 weeks (F\textsubscript{6,1}= 4.3; P=0.1). Administration of exogenous testosterone resulted in an increase in testosterone concentration after 4 weeks (F\textsubscript{6,1}=0.28; P=0.059). A further increase in testosterone concentration occurred after 8 weeks in rats administered testosterone when compared to testosterone-treated control rats (F\textsubscript{6,1}=3.2; P=0.02). No significant difference in the levels of testosterone was observed in the rats killed 8 weeks after irradiation when compared to the rats killed after 4 weeks in this group of rats exposed to 6.0 Gy \(\gamma\)-irradiation (F\textsubscript{6,1}= 3.62; P=0.23).
4. 5. DISCUSSION

Following testosterone administration, the mean concentration of LH in the testosterone treated control group became lower compared to the non-testosterone treated control. Exogenous testosterone led to the suppression of the synthesis and subsequent release of LH by the gonadotropes in the anterior pituitary. This in turn resulted in a decreased concentration of LH in the circulation through negative feedback.

Exposure of rats to 3.5 Gy gamma irradiation resulted in a slight but significant increase in plasma LH concentration. Kurdoglu et al. (1996) similarly found LH concentration to increase 10 weeks post exposure of the rat testes to 6.0 Gy gamma irradiation. However, Kurdoglu et al. (1996) reported that LH levels increased by 200%, whereas in this study LH concentration increased by only 20%. Despite the fact that Kurdoglu et al. (1996) allowed a greater time (10 weeks compared to 8 weeks in this study) prior to measuring LH concentrations, it is highly unlikely that increases of the same order of magnitude could be observed in this study if the concentrations had been measured two weeks later, since no significant difference was found in the concentrations of LH between rats killed 4 weeks and those killed 8 weeks after irradiation.

Nonetheless, results in this study were consistent with the findings of Pinon-Lataillade et al. (1983). They exposed Sprague Dawley rats to 6.4 Gy irradiation, and observed a 25% increase in LH concentration after 7 weeks. According to Pinon-Lataillade et al. (1993) an increase in LH concentration is usual after severe testicular damage.
Wang et al. (1993) also observed a slight but significant increase in LH concentration 7 weeks following the exposure of rats to 5.3 Gy gamma irradiation. The elevated concentrations of LH have not been explained in the literature, however the circulating testosterone concentration is not significantly altered following exposure of rat testes to irradiation and this may explain the results. Elevated testosterone concentration in the circulation inhibits the production of LH (figure 4.1) through negative feedback pathways.

Indeed, testosterone administration lowered the concentration of LH in the 3.5 Gy dose group, but interestingly did not bring about a concomitant drop in LH concentrations in the 6.0 Gy dose group. This may be due to increased tubule damage resulting from the high dosage of irradiation used.

There was a dramatic rise in the mean plasma FSH after exposure of the rats to an irradiation dose of 6.0 Gy. Interestingly, Huckins et al. (1978) reported that FSH levels were unaffected by gamma irradiation doses between 0.2-7.0 Gy. But Kurdoglu et al. (1994) reported a 50% increase in plasma FSH 10 weeks after exposure to 6.0 Gy γ-irradiation. In this study plasma FSH exhibited a 45% increase 8 weeks post irradiation. Increased FSH concentrations have also been reported by Pinon-Lataillade et al. (1985), Pineau et al. (1989) and Kangasniemi et al. (1996b). Pinon-Lataillade et al. (1985) and Pineau et al. (1989) all reported greater increase in FSH concentration than LH concentration.
Enhanced FSH secretion may be attributed to germ cell loss. Previous studies have shown conclusively that FSH concentrations increase with a reduction of pachytene spermatocytes (Pinon-Lataillade et al., 1985). Exposure of rat testes result in a dose dependent reduction of spermatogenic cells, which include spermatocytes (histology results-chapter 2). It is plausible that these cells may play a role in the regulation of inhibin production by the testis that regulates the secretion of FSH (Sharp et al., 1981; Sharp, 1986; Meistrich et al., 1996).

Pachytene spermatocytes provide the Sertoli cells with an early signal for inhibin production (Pinon-Lataillade et al., 1985). Pineau et al., (1989) has further suggested that there is strong evidence for a paracrine interaction between pachytene spermatocytes and Sertoli cells for inhibin secretion.

The FSH concentration in the 3.5 Gy irradiated and testosterone treated rats did not differ significantly with FSH levels in rats that received no testosterone. It is noteworthy that testes of these testosterone treated rats were repopulated with spermatogenic cells (chapter 2). This may imply that the high levels of FSH following irradiation are not responsible for the inability of surviving stem cells to divide and repopulate the seminiferous tubules.

Plasma testosterone levels following exposure to irradiation did not differ significantly to those of the control in both dose groups. However there was a significant increase in the testosterone treated control.
In previous studies, no significant changes in plasma testosterone concentration were observed following exposure of the rats to low dose gamma irradiation (Cunningham and Huckins, 1978; Pineau et al., 1989; Wang et al., 1983; Kurdoglu et al., 1996).

Pinnon-Lataillade et al. (1985) did not observe changes in plasma testosterone nor in the weights of testosterone-dependent accessory sex organs following exposure to low dose gamma irradiation.
CHAPTER 5. CONCLUSION

Radiotherapy administered on its own or in combination with chemotherapy to treat various cancers has proven highly effective (Mc Laughlin et al., 1991; Kurdoglu et al., 1994). However this form of treatment results in the side effect, azoospermia, with normo-spermia rarely if ever being regained in patients (Kurdoglu et al., 1994). In this study, the effects of low dose $\gamma$-irradiation on the rat testis were studied. The possibility of reversing these effects using exogenously administered testosterone was investigated.

The effects of low level $\gamma$-irradiation on testicular histology, epididymal sperm motion, and on the primary reproductive hormones in Sprague Dawley rats, and their subsequent reversal employing hormone treatment have not been undertaken before. Hence this represents the first documented comprehensive study.

Sprague Dawley rats were subjected to two low dose $\gamma$-irradiations (3.5 and 6.0 Gy). Irradiation led to a dose-dependent reduction in spermatogenic cell numbers, which occurred concomitantly with a decrease in the testicular index. Spermatogenic cells have been termed the radiosensitive tissue within testis (Wang et al, 1993), and constitute some 70% of the normal testicular weight (Potten and Henry, 1983). Therefore it seems logical that germ cell loss would lead to a decrease in testis mass, and subsequently an increase in testicular index.

Following 3.5 Gy irradiation, percentage sperm motility was reduced, and two kinematic parameters, VCL and VSL, also decreased.
A dose of 6.0 Gy resulted in a depletion of spermatogenic cells, and decapitation of epididymal sperm. These clearly physical and direct effects of irradiation on epididymal sperm could not be reversed by testosterone administration during the prescribed period of treatment.

Testosterone administration induced recovery of repopulation indices in rats exposed to 3.5 Gy irradiation. Furthermore, there was a reduction in testicular index towards the levels seen in control animals, indicating that testicular mass had been restored towards normal levels. Increasing the time of exogenous testosterone administration from 4 to 8 weeks yielded a higher recovery.

However, recovery was not paralleled in the sperm motility study. Firstly, irradiation brought about physical damage to the epididymal sperm. It is logical that testosterone treatment would not repair the damaged sperm. Secondly, even though spermatogenic cells repopulated the tubules, increased percentage motility of epididymal sperm was not observed. It is hypothesized that this may have been due to insufficient time-period for recovery.

It may take up to 4 cycles for spermatogonia to mature into spermatozoa, with each cycle taking approximately 12 days (Meistrich and van Beek, 1993). Epididymal transit takes approximately 16 days. Therefore the maximum recovery period of 8 weeks used in this study may have been sufficient for the recovery of spermatogenesis, but was probably not enough to observe improvement or changes in epididymal sperm motility parameters.
Circulating plasma LH and FSH concentrations increased following exposure to 3.5 Gy irradiation. The increase was more pronounced in the 6.0 Gy dose group. Plasma testosterone, however, showed fluctuations that were statistically not significant in both dose groups.

The increase in LH has been associated with destruction of the tubules. The increase in FSH concentration has been attributed to the destruction of spermatocytes, and histological findings revealed destruction of the various spermatogenic cell populations, including spermatocytes.

Testosterone treatment led to a reduction in the circulating LH levels. The reduction in LH concentrations may have contributed to recovery by lowering the intratesticular testosterone levels (ITT). A reduction in ITT has been cited as a consistent feature of treatment modalities that leads to recovery of spermatogenesis following exposure of the testes to γ-radiation.

The concentration of FSH was not restored to control levels following testosterone treatment. However, it is speculated that the recovered spermatocytes may have been able to provide the signal for inhibin production if more time had been allowed, ultimately resulting in a return to normal FSH concentrations. In previous studies conducted on rats exposed to 3.5 Gy γ-irradiation, the use of the GnRH-agonist, Zoladex, resulted in a return of FSH to values similar to control levels only after 20 weeks (Meistrich et al., 1997). It is speculated that if more had been allowed, the recovered spermatocytes would have provided the signal for inhibin production, ultimately resulting in a return to normal FSH concentrations.
Therefore, testosterone resulted in an increase in testicular weight towards control levels, restoration of repopulation indices (RI) and testicular indices, and the repopulation of the seminiferous tubules by spermatogenic cells at various stages of division. According to Wilson et al. (1999), restoration of RI and testicular weight are indicative of spermatogenic recovery. Therefore testosterone administered exogenously following exposure of rats to low dose gamma irradiation aids in the recovery of spermatogenesis. The focus of future studies should be to conduct a testosterone dose dependent investigation to identify the most effective dose for optimal results.
REFERENCES


