INVESTIGATING THE EFFECTS OF EXERCISE ON INITIATION OF NITROSOMETHYLUREA INDUCED MAMMARY TUMORIGENESIS IN YOUNG RATS

by

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Investigating the effects of exercise on initiation of nitrosomethylurea induced mammary tumorigenesis in young rats

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Abstract

It has been suggested by epidemiological studies that exercise during the college years decreases the risk of breast cancer. The susceptibility of the mammary gland to carcinogenic attack depends on many factors including: 1) developmental stage of the mammary gland; 2) proliferation rate of epithelial cells; and 3) DNA damage. The purpose of this thesis was to assess the effects of exercise in young female Sprague Dawley rats on mammary gland development, carcinogen distribution, binding and damage,and subsequent tumor development.

Rats were run on motorized treadmills for four weeks (from twenty one to fifty days of age). Exercise had no effect on mammary gland development, although exercise significantly increased the proliferation rate of the mammary gland epithelial cells. The increased proliferation rate did not affect the amount of Nitrosomethylurea (NMU)- induced DNA damage as indicated by the nick translation assay. Increasing the amount of NMU injected (25, 37.5 or 50 mg/kg) resulted in a dose response in terms of NMU in both the liver and serum. This relationship was apparent at the mammary gland DNA level. However, DNA damage did not increase with increasing doses of NMU. When individual glands were examined, abdominal mammary glands had a lower incorporation of NMU than thoracic or inguinal glands for any concentration of NMU. Of particular interest was the finding that DNA damage was also signficantly lower in the abdominal mammary glands.

We tested the effects of exercise prior to carcinogen administration on subsequent tumor development at two doses of NMU. At the higher dose (50 mg/kg) exercise significantly lowered the tumor multiplicity. However at the lower dose (37.5 mg/kg) this effect was

not seen. At either dose there was no effect on incidence or latency. At a dose of 37.5 mg/kg, exercise significantly increased the growth rate and final tumor weights. Due to these differences in growth rates and final tumor weights we assessed the amount of insulin-like growth factor I receptor and estrogen receptor proteins in exercised and sedentary tumors as well as in normal mammary gland tissue. There was a significant increase in the content of both receptors in the tumors relative to the normal tissue. There was a significant difference in receptor content between tumors derived from exercised and sedentary rats when expressed per milligram tissue. This difference was lost when the receptor content was expressed per microgram protein.

This thesis suggests that exercise prior to carcinogen administration does effect tumor outcome in a somewhat dose dependent manner. Although exercise increases the proliferation rate of mammary epithelial cells there is no effect on DNA damage or final tumor outcome. This suggests that other mechanisms must compensate for the increased proliferation.

Dedication

This thesis is dedicated to the fight against breast cancer.

Acknowledgments

I would like to thank my supervisor Dr. Wade Parkhouse for his support and for understanding my wanting to undertake this project. I gained a vast amount of knowledge during my time in his lab. I would also like to thank Dr. Joanne Emerman and Dr. Miriam Rosin for their insight and support.

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Chapter I Review of literature

Breast cancer is the leading cause of death among women in western countries. In North America one in nine women will develop the disease. It is a physically and psychologically debilitating disease. The drain on the health care system is continually increasing. Despite early screening and medical advances the incidence rate is not declining. Epidemiological studies on breast cancer have been extensive, however definitive etiologic factors are not known. The risk factors involved in breast cancer tend to be associated with the reproductive history of the individual, which is correlated to the stage of mammary gland development and the exposure to hormones such as estrogen and progesterone. Approximately five percent of breast cancers are genetic whereas the other ninety five percent are sporadic. The current trend in cancer research is to look at ways of preventing the disease. Lifestyle factors such as diet and exercise have emerged as potential preventative agents for this disease.

1.1 Histopathology of breast cancer

The World Health Organization has classified breast tumors into the following categories (Cotran et al. 1989):

A. Noninvasive (noninfiltrating)

1. intraductal carcinoma
2. intraductal papillary carcinoma
3. lobular carcinoma in situ

B. Invasive

1. invasive ductal carcinoma

2. invasive lobular carcinoma
3. medullary carcinoma
4. colloid carcinoma
5. Paget's disease
6. Tubular carcinoma
7. adenoid cystic carcinoma
8. invasive comedocarcinoma
9. apocrine carcinoma
10. invasive papillary carcinoma

The most common types of infiltrating breast tumors along with the incidences are outlined below (Cotran et al. 1989). 70-90% of invasive carcinoma is typically of ductal origin.

histologic type	incidence (%)
invasive duct carcinoma	
-pure	52.6
-combined with other types	22.0
medullary carcinoma	6.2
colloid carcinoma	2.4
Paget's disease	2.3
other pure types	2.0
other combined types	1.6
infiltrating lobular carcinoma	4.9
combined lobular and ductal	6.0

1.2 Epidemiology and risk factors

From epidemiological studies, researchers have determined that breast cancer is two diseases, one that occurs premenopausally and the other is postmenopausal in origin (Zumoff 1988). It is important when looking at epidemiological studies to remember that the relative contributions of genetic and environmental factors may be different for the two diseases. The established risk factors for breast cancer include family history of breast cancer, early menarche, late age at first childbirth, late age at menopause, history of benign breast disease and exposure to ionizing radiation (Harris et al. 1992). Obesity is a risk factor in postmenopausal women, however in premenopausal women reduced incidence is associated with obesity (Harris et al. 1992). Estrogenic stimulation increases risk (Pike et al. 1983) which may explain the role of age at menarche and menopause, and the obesity among postmenopausal women as risk factors. These factors are involved in the production of estrogen with early menarche and late menopause increasing the lifetime exposure to estrogen. In postmenopausal women, estrogen is produced by the conversion of androgens in fat tissue. Early pregnancy has a protective effect which may result from pregnancy induced differentiation of breast cells (Russo et al. 1982). The ability of a carcinogen to bind to DNA and cause damage is due to the presence of the undifferentiated, highly proliferating terminal end buds. It is these structures that are highly susceptible to carcinogenic insult (Russo and Russo 1987).

Differences in breast cancer rates among countries (Armstrong and Doll 1975) and increased incidence rates among immigrants from countries with low incidence to those with high incidence (Buell 1973) suggest the involvement of nongenetic factors in breast cancer (Harris et al. 1992). The overall incidence of breast cancer in Japan is one-fifth that of the United States (Armstrong and Doll 1975). Age at menarche may partially account for international differences, average age of menarche in China is 17 years (Chen et al. 1990)

vs. 12.8 years in the United States (Wyshak et al. 1982). Another explanation may be the differences in diet. Typically North Americans eat a much higher fat diet. In animal models caloric restriction and decreased fat consumption reduced incidence of mammary tumors (Welsch 1992; Ip 1990).

1.3 Etiology of breast cancer

Possible etiologic factors for breast cancer have been elucidated from epidemiological findings and animal studies. These factors can be divided into three groups: genetic factors, hormonal imbalance and environmental influences. The genetic factors involved in familial breast cancer are not known at this time, however the *brca1* and *brca2* genes are likely candidates (Ford and Easton 1995). Growth promoting proto-oncogenes and growth constraining tumor suppressor genes regulate the proliferation of normal cells (Weinberg 1991). Oncogenes are caused by mutations that activate proto-oncogenes and force the growth of tumor cells (Weinberg 1991). Conversely, cells are liberated from the constraints imposed by tumor suppressor genes when genetic lesions inactivate these genes. This leads to unconstrained growth of the cells (Weinberg 1991). Oncogenes and tumor suppressor genes that may be involved in sporadic breast cancer include c-erb-2, p53, TGF-α (Davidoff et al. 1991; Maguire et al. 1992; Parham and Jankowski 1992).

Long duration of reproductive life (i.e. time between menarche and menopause), nulliparity and late age at first child have been identified as risk factors. Each of these factors result in increased exposure to estrogen peaks during the menstrual cycle, which implies estrogen may play a role in breast cancer development. There are two mechanisms which are thought to be involved: 1. breast cells have estrogen receptors through which estrogen may have a direct mitogenic effect; and 2. autocrine/paracrine production of growth factors is

estrogen dependent and these growth factors are believed to be involved in the progression of breast cancer (Miller 1990). Estrogen is just one of the hormones thought to be involved in breast cancer. Other hormones may be involved either directly or indirectly.

Two environmental factors that appear to be involved in breast cancer are diet and exposure to radiation (Harris et al. 1992). Other factors that are not as well established include smoking and alcohol consumption (Harris et al. 1992).

Several hypotheses have been put forth to try and explain the hormonal involvement in breast cancer (Zumoff 1988). These hypotheses are outlined below.

I. Adrenal androgen insufficiency hypothesis

A number of studies have identified a deficiency of adrenal androgen in women with premenopausal breast cancer (Zumoff 1988). The onset of disease is preceded by the decreased production and subnormal plasma levels of the adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). This deficiency may play a causal role in susceptibility to premenopausal breast cancer. DHEA administered to a strain of mice with a high incidence of the disease prevented the development of breast cancer (Schwartz 1979).

II. Ovarian androgen excess hypothesis

Chronic anovulation is characteristic of premenopausal breast patients and excessive urinary excretion of testosterone and androstenediol is seen in both the pre- and postmenopausal patient (Zumoff 1988). Ovariectomy results in a return to normal androgen excretion levels and is effective in preventing recurrence of breast cancer (Zumoff 1988). Chronic anovulation is known to be associated with increased ovarian androgen production, and ovarian dysfunction is an established risk factor for breast cancer (Coulam et al. 1983).

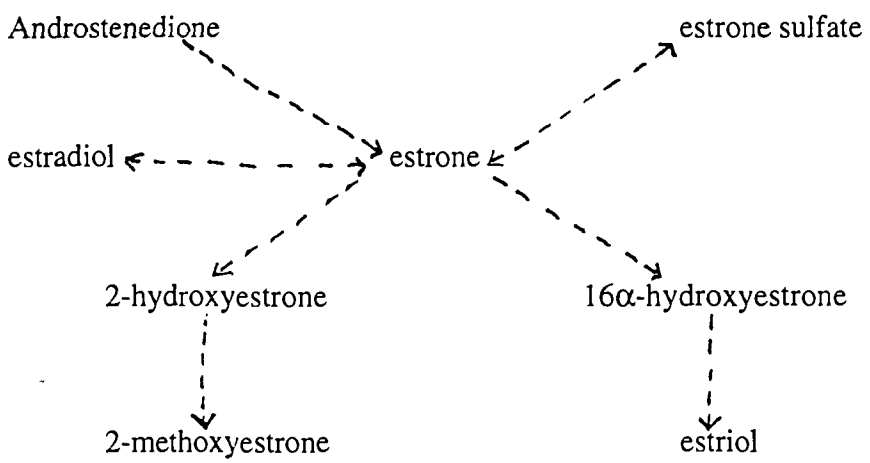
This suggests that ovarian androgen excess may be a factor in breast cancer in addition to increased ovarian estrogen production.

III. Prolactin hypothesis

Prolactin is a mammotrophic hormone making it a possible candidate for involvement in breast cancer. Prolactin excess has been linked to mammary cancer in experimental animals, however its role in human cancer has not been established (Zumoff 1988). Some studies have shown elevated levels of prolactin in both pre-and postmenopausal patients (Rose and Pruitt 1981) whereas others have reported decreased nocturnal serum prolactin levels and normal daytime levels in postmenopausal patients and elevated nocturnal prolactin levels in premenopausal breast cancer patients (Malarkey et al. 1977). Musey et al. reported that a decrease in serum prolactin levels was observed after a first full term pregnancy (Musey et al. 1987). This may help to explain the protection early pregnancy confers against breast cancer.

IV. Estradiol 16α-hydroxylation hypothesis

The estradiol metabolism pathway:



The 2-hydroxylation pathway leads to nonestrogenic metabolites, whereas the 16α hydroxylation produces two highly estrogenic metabolites. The ratio of the two pathways determines the total estrogenic impact of a given amount of secreted estradiol. This ratio is constant under normal biological circumstances. Increased 16α-hydroxylation is found in: 1) women with breast cancer; 2) women at high risk for familial breast cancer; and 3) mouse strains with high incidence of breast cancer (Zumoff 1988).

1.4 Natural history of breast cancer

Breast cancer is considered to be a systemic disease. At the time of initial diagnosis, two-thirds of all patients have metastases to lymph nodes. Spread of the tumor can occur through the lymphatic system and the circulatory system. Figure 1.1 represents the positions of the lymph nodes and the possible directions of spread. The two favored directions of drainage are the axillary nodes and the nodes along the internal mammary artery. The pattern of nodal spread is heavily influenced by the location of the cancer as outlined in figure1.2. The size of the tumor is closely associated with the presence of axillary metastases. Distant metastases can affect any site in the body but the most common are the lungs, bone and liver.

tumor size (cm)	patients with four or more positive nodes (%)
<1	25
1-2	35
2-3	50
>3	55-65

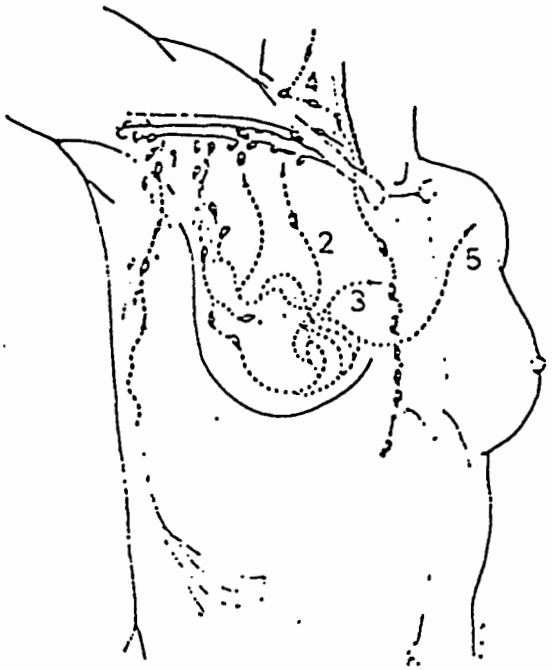


Figure 1.1. Lymphatic drainage of the breast to regional node groups. 1. main axillary groups. 2. interpectoral node leading to apex of axilla. 3. internal mammary group. 4. supraclavicular group. 5. lymphatic channels opposite axilla.

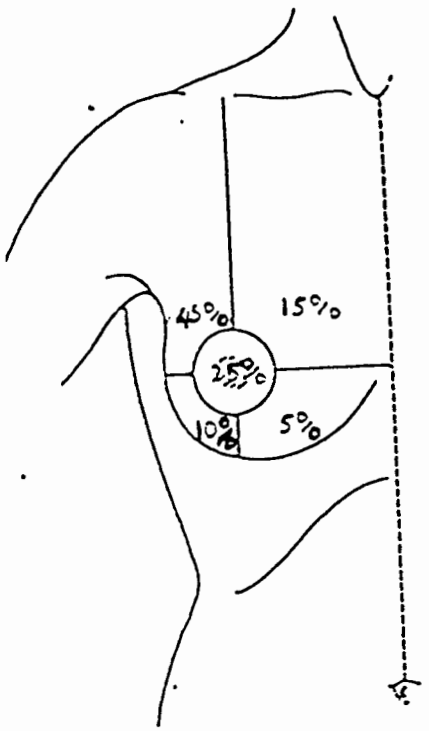


Figure 1.2. Regional locations of tumors in the breast.

1.5 Diagnosis and staging systems

Early screening for breast cancer has been a major initiative in the health care field. Monthly self breast examinations as well as regular examinations by a physician are strongly recommended. Mammograms are recommended every two years in women forty to fifty years of age, and annually after 50. A biopsy will confirm the presence of malignant cancers and allow histological examination and determination of estrogen receptor status of the tumor. The clinical stage of the tumor is classified according to the TNM system (American Joint Committee on Cancer 1988). T represents the tumor size, N is the lymph node involvement and M is the presence of distant metastases. Progressing from stage I to III there is increased size of tumor and a greater number of positive lymph nodes. Stage IV involves distant metastases.

1.6 Prognostic factors of breast cancer

The single most important prognostic factor is axillary node status. The greater the number of lymph nodes involved the poorer the survival rate. Other factors include clinical stage, tumor size and receptor status. With increasing tumor size and advancement in clinical stage, there is a concomitant decline in survival rate. Estrogen receptor negative patients are more likely to be younger (premenopausal) and have rapidly growing tumors that tend to recur earlier and to cause death earlier.

1.7 Management/treatment of breast cancer

The first step of intervention with breast cancer is surgery . The current practice is to leave the breast as intact as possible for cosmetic reasons. The surgery is followed by radiation therapy especially for locally advanced cancer. Adjuvant therapy is an important mode of management in breast cancer and can be in the form of either chemotherapy or hormone therapy. Adjuvant treatment is defined as therapy administered to patients with no demonstrable residual tumor after the initial treatment. The current consensus is that in women with lymph node involvement, chemotherapy should be given to premenopausal patients and hormone therapy to those who are postmenopausal. Chemotherapy is especially important for those patients with metastases. Cytotoxic drugs such as cyclophosphamide, methotrexate and fluorouracil are used with high risk patients. Chemotherapy treatment is used for metastatic breast cancer if: 1) visceral metastases are present; 2) hormonal treatment is unsuccessful or the disease has progressed after an initial response to hormonal manipulation; or 3) the tumor is estrogen receptor negative. Hormone treatment involves administering antiestrogens such as tamoxifen or drugs that will block synthesis of hormones such as aminoglutethamide. The tumor must be estrogen receptor positive for hormone therapy.

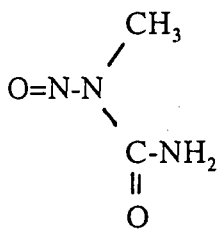
1.8 Animal models for mammary carcinogenesis

The two most common carcinogens used to induce mammary tumors in rats and mice are 7,12 dimethylbenz[a]anthracene (DMBA) and N-nitrosomethylurea (NMU). The two share common features such as organ site specificity, reliability of tumor induction, tumors of ductal origin, tumors of predominantly carcinomatous histopathology and tumors of varying hormone responsiveness (Welsch 1985). However, there are a number of

differences between the two models. DMBA is a procarcinogen which requires metabolic activation by the host whereas NMU is direct acting (Welsch 1985). DMBA tumors are more prolactin dependent whereas NMU tumors are more estrogen dependent (Welsch 1985). For example, in rats bearing DMBA induced tumors, but not NMU tumors, estrogen does not stimulate tumor growth when serum prolactin levels are suppressed (Manni et al. 1977). Prolactin suppressing drugs have more of an antitumor effect on DMBA mammary tumors than NMU mammary tumors (Rose and Noonan 1982). NMU tumors are more aggressive and locally invasive (Welsch 1985). The number of benign tumors induced by NMU is lower than in the DMBA model (Welsch 1985).

1.9 Nitrosomethylurea (NMU)

Nitrosomethylurea (NMU) is a direct acting alkylating agent classified as a nitrosoamide with the following structure.



The spontaneous decomposition of NMU produces electrophiles which attack nucleic acids forming methylated nucleosides. In 1975, mammary carcinomas were induced in female rats with monthly intravenous (iv.) injections of NMU (Bots and Willighagen 1975; Gullino et al. 1975). Subsequently, the procedure for tumor induction was modified by Moon et al. (1977) with two iv. injections one week apart. The current protocol for inducing mammary carcinomas with an iv. injection of NMU requires one injection typically at a dose of 50 mg NMU/kg around 50 days of age (McCormick et al. 1981).

Thompson et al. have further modified the procedure by using a single intraperitoneal (ip.) injection arguing that technically ip. injections are easier to administer than iv. injections (Thompson and Adlakha 1991). The tumor incidence is comparable with iv. and ip. injections (Thompson and Adlakha 1991). Several studies using both multiple and single injections have demonstrated a dose response relationship between NMU dose and tumor incidence (Verdeal et al. 1982; McCormick et al. 1981; Thompson and Adlakha 1991; Anisimov 1988). Tumor incidence and multiplicity increase with increasing NMU dose whereas latency increases with decreasing NMU dose. Hypophysectomy causes rapid tumor regression, but administration of prolactin or estrogen to these rats stabilizes tumor growth (reviewed in Welsch 1985). At or after NMU administration, ovariectomy inhibits development and growth of tumors (reviewed in Welsch 1985). Administration of antiestrogens also inhibits development and growth of tumors (reviewed in Welsch 1985). Progesterone in moderate doses shows an inhibitory effect if it is administered ten days after or seven days before and continuing after NMU treatment (reviewed in Welsch 1985). However, it shows a stimulatory effect when administered at the time of NMU treatment (reviewed in Welsch 1985). Pregnancy and lactation commencing after NMU suppresses tumor development (reviewed in Welsch 1985). Decreased incidence occurs when pregnancy without lactation follows NMU treatment: latency period of mammary tumor appearance significantly decreases in pregnant animals. Reduced latency suggests that the hormonal milieu of pregnancy accelerates growth of certain NMU induced mammary cancers. Receptors for estrogen, progesterone, androgens and prolactin have been detected and examined in NMU induced rat mammary cancers (reviewed in Welsch 1985).

Administration of NMU activates the Ha ras oncogene in the rat mammary gland (Sukamar et al. 1983; Zarbl 1985; Kumar 1990). The gene is activated by a G to A transition in the second nucleotide of codon 12. The major product of NMU alkylation is methylguanosine. The presence of methylguanosine will cause a G to A transition in an amino acid sequence.

1.10 DNA damage and DNA repair

DNA damage can result from radiation and/or chemical carcinogens. Chemical carcinogens can be either direct acting or procarcinogens which require host activation. Below are the types of DNA damage that can occur in a cell (Tannock and Hill 1992).

Nucleotide level:

-DNA carcinogen adduct formation

-base oxidation

-base loss

DNA molecule level:

-single strand break

-double strand break

-intrastrand crosslink

-interstrand crosslink

-DNA protein crosslink

Chromosome level:

-chromosome deletions

-chromosome translocations

-loss of chromosome

Alkylating agents such as NMU are electrophilic compounds which alkylate DNA. 7-methylguanine and O^6 methylguanine are the major methylated products resulting from NMU administration. Repair of this damage can occur in one of two ways: 1) removal of alkyl groups by alkyltransferase or 2) removal of altered base by a glycosylase creating an apurinic or apyrimidinic site that can be repaired by excision repair which may occur at the base or nucleotide level (Myles and Sancar 1989). Direct repair of the modified base occurs

when the alkyl group is transferred from the base to the alkyltransferase (Tannock and Hill 1992). Nucleotide excision repair involves a complex multi enzyme system involved in a stepwise process as follows: 1) recognition of damage; 2) nicking of damaged DNA strand next to damaged nucleotide by an endonuclease; 3) removal of damaged region by exonuclease; 4) resynthesis of DNA by polymerase using opposite intact strand as template; and 5) ligation of single strand gap resulting from polymerase reaction (Mullaart et al. 1990).

Methods of detecting DNA damage and/or repair include the alkaline unwinding assay, unscheduled DNA synthesis (UDS), the and nick translation assay. UDS is a classic test for DNA damage (Snyder and Matheson 1985). However, negative test results occurred with many DNA damaging carcinogens (Snyder and Matheson 1985). The sensitivity of the assay is brought in to question when considering agents that induce very low levels of damage or damage that requires little or no resynthesis during repair (Snyder and Matheson 1985). The nick translation assay was modified to circumvent these problems (Snyder and Matheson 1985). The nick translation procedure can assess both DNA damage (appearing as strand breaks) and DNA repair. The assay involves the introduction of exogenous DNA polymerase I and deoxynucleotide triphosphates (dNTPs) into permeabilized cells. One of the dNTPs is labeled with tritium (^{3}H). The exogenous *E. coli* DNA polymerase I binds to free 3' OH termini and in the presence of dNTPs translates the nick linearly along the DNA helix. This is accomplished by the 5' to 3' exonuclease activity and polymerizing activity present on the same enzyme molecule (Kelly et al. 1970).

1.11 Susceptibility of the rat mammary gland to carcinogenic attack

The highest incidence of carcinogen induced mammary carcinomas occurs when the rats are injected around fifty days of age, a time when the number of terminal end buds (TEBs) and the level of DNA synthesis are at their highest (Russo and Russo 1982). The susceptibility of a cell to undergo malignant transformation depends on factors such as rates of DNA synthesis, cell proliferation, the length of G_1 and S phases of the cell cycle and the cellular competency in DNA repair (Russo and Russo 1982). Rapid cell division increases the ability of the cell to bind to a carcinogen and a short cell cycle allows less time to repair the damage to the DNA (Russo and Russo 1987). The cell cycle in TEBs is considerably shorter than that of alveolar buds (Abs) as the G_1 phase of the cell cycle is shortened in TEBs. G_1 represents the time between mitosis and the beginning of DNA replication (Villee 1985). During this phase the cell grows and there is increased activity of the enzymes involved in DNA synthesis and repair (Villee 1985). Thus a major difference between TEBs and ABs is that TEBs are cells possess a higher proliferative activity (Russo and Russo 1987).

1.12 Mammary gland anatomy and development

Russo and Russo have extensively characterized the structure of the mammary gland (1987). The mature mammary gland is composed of a system of 15 to 20 lactiferous ducts that lead into a main lactiferous duct (figure 1.3). The ducts end in structures known as lobules which are composed of acini, the secretory cells of the mammary gland. At puberty the ductal system starts to form. These immature ducts end in terminal end buds (TEBs). It is these structures that eventually give rise to the lobules. With the onset of menstruation, the lobular structures begin to appear composed of the more differentiated alveolar buds

(ABs). With each successive menstrual cycle the number of lobules increases. Three types of lobules have been identified. They differ in the number of alveolar buds/lobule and in their proliferation index such that type I is the least dense but possesses the highest rate of proliferation. Type I is typically found in nulliparous women whereas type II and III are common in women who have had children. The ducts and ductules (lobules) are enclosed in a loose, delicate myxomatous stroma that contains a scattering of lymphocytes (intralobular connective tissue) . The individual lobules are enclosed within a denser, collagenous, fibrous interlobular stroma. At puberty the mammary gland consists almost entirely of stroma. The ratio of parenchyma:stroma increases with puberty and during pregnancy parenchyma contributes to about 73% of the gland.

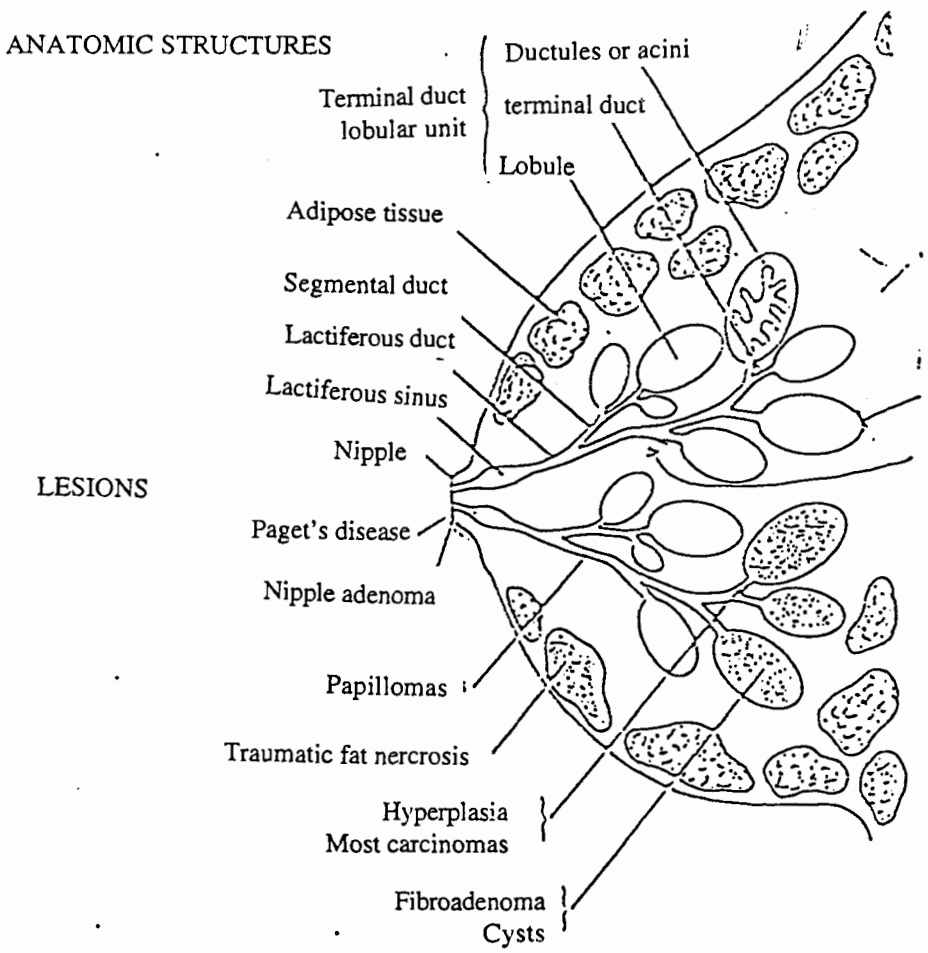
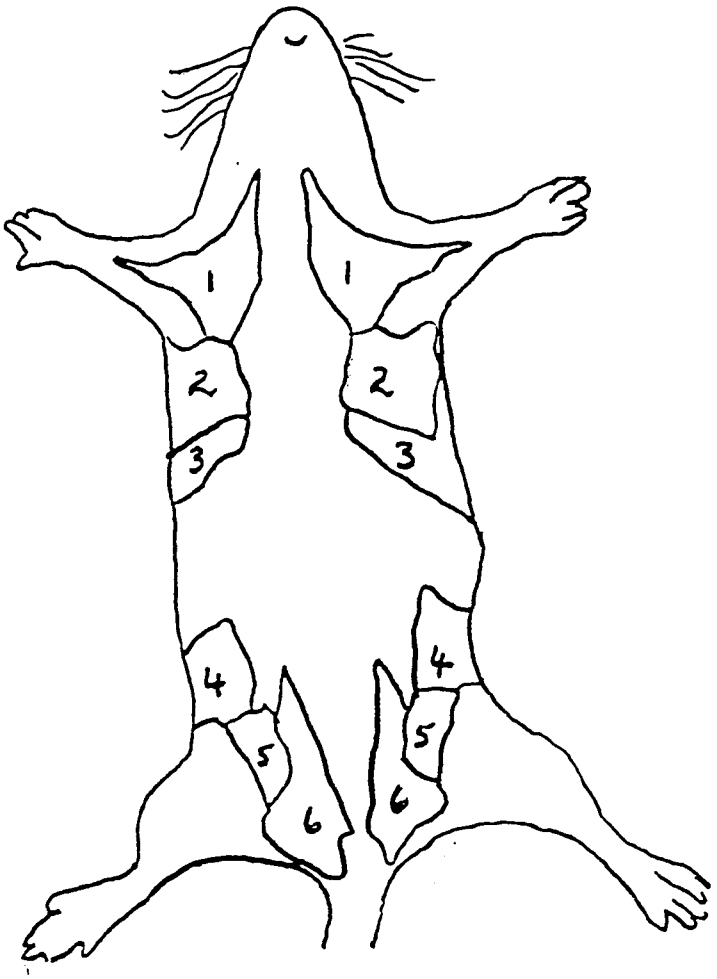


Figure 1.3. Anatomy of the breast.



1= cervical mammary glands

2,3= thoracic mammary glands

4,5= abdominal mammary glands

6= inguinal mammary glands

Figure 1.4. Mammary glands in the rat

In the rat there are six pairs of mammary glands (figure 1.4). The structure and development of the rat mammary gland is similar to that of the human. At 21 days of age, the TEBs begin to differentiate into the smaller ABs which are lined by a two layered epithelium (Russo and Russo 1987). The rats estrous cycle starts between 35 and 42 days old and with each cycle the differentiation is enhanced. Once the animal reaches sexual maturity (6-8 weeks of age) the mammary ductal tree completely fills the mammary fat pad, with characteristic ductal spacing being maintained as long as the animal remains nonpregnant (Topper and Freeman 1980). Little growth occurs until pregnancy ensues (Topper and Freeman 1980).

The type of cell(s) found in the mammary gland depend on the location (Cotran et al. 1989). The areola, nipple and mouths of the main lactiferous ducts are composed of stratified squamous epithelium . As you move down the main duct the cells are identified as pseudo-stratified columnar epithelium. As the main duct begins to branch off the ducts are lined with a double layer of cuboidal epithelium and as the branches become smaller and smaller the cells become single layered. The smaller ducts and ductules also have a layer of myoepithelial cells. The myoepithelial cells contain myofilaments running parallel to the long axis of the duct. These myofilaments contract causing the milk to be secreted from the acini cells.

1.13 Hormones and mammary gland development

A complex of hormones regulate the growth and development of the mammary gland (reviewed by Topper and Freeman 1980; Borellini and Oka 1989; Imagawa et al. 1990). Classical studies on hormones were based on three experimental designs: 1) effect of removal of endocrine glands; 2) administration of hormones to intact animals; and 3)

hormone replacement therapy in endocrinectomized hosts (Imagawa et al. 1990). General conclusions drawn from such studies are outlines below (Topper and Freeman 1980; Borellini and Oka 1989; Imagawa et al. 1990). Estrogen and either growth hormone or prolactin are the minimal requirements for ductal growth, however neither hormone is effective alone. Progesterone is not required for ductal growth. Maintenance of ductal structures is hormonally different than the requirement for ductal growth. Provided either adrenal or ovarian secretion is present, pituitary hormones are not required. Lobuloalveolar development during pregnancy is dependent on estrogen, prolactin and progesterone. Progesterone levels increase early in gestation and is probably the initiator of lobuloalveolar development. Progesterone or progesterone and estrogen together stimulate DNA synthesis in both end buds and surrounding ducts whereas estrogen causes synthesis only at the tips of the end buds. Progesterone receptor concentration varies with developmental state of the mammary gland. Receptors are abundant in virgin mice, reduced during pregnancy and nonexistent during lactation. Estrogen causes increase in progesterone receptor number. Progesterone stimulates ductal branching and lobuloalveolar development, while inhibiting lactogenesis during pregnancy. The number of prolactin receptors is inversely related to the serum levels of progesterone. Prolactin and growth hormone are the anterior pituitary factors responsible for functional differentiation.

Recent *in vitro* studies have shown progesterone and prolactin are mammogenic hormones that stimulate growth, whereas estrogen has no direct effect on growth (reviewed in Imagawa et al. 1990). Several theories as to how estrogen affects the mammary gland have been put forth. *In vivo* estrogen may act in one of three ways: 1) as a primary mitogen in mammary tissue; 2) as a permissive agent that sensitizes mammary tissue to the action of lactogenic hormones and growth factors; or 3) act indirectly through stimulation of secretion of other hormones and growth factors. Estrogen could potentiate the effect of

progesterone and prolactin on postpubertal ductal growth and alveolar development by stimulating pituitary prolactin secretion and inducing progesterone receptors.

1.14 Exercise and menarche

The menstrual cycle can be defined in terms of ovarian events. The follicular phase is considered the proliferative phase of the cycle and is characterized by the development of a mature ovarian follicle which contains the ovum or egg. The luteal phase is defined as the secretory phase because the corpus luteum produces and secretes progesterone and estrogens. Plasma hormone concentrations and ovarian events are depicted in figure 1.5. The hypothalamic-pituitary ovarian axis is responsible for the regulation of the menstrual cycle (figure 1.6).

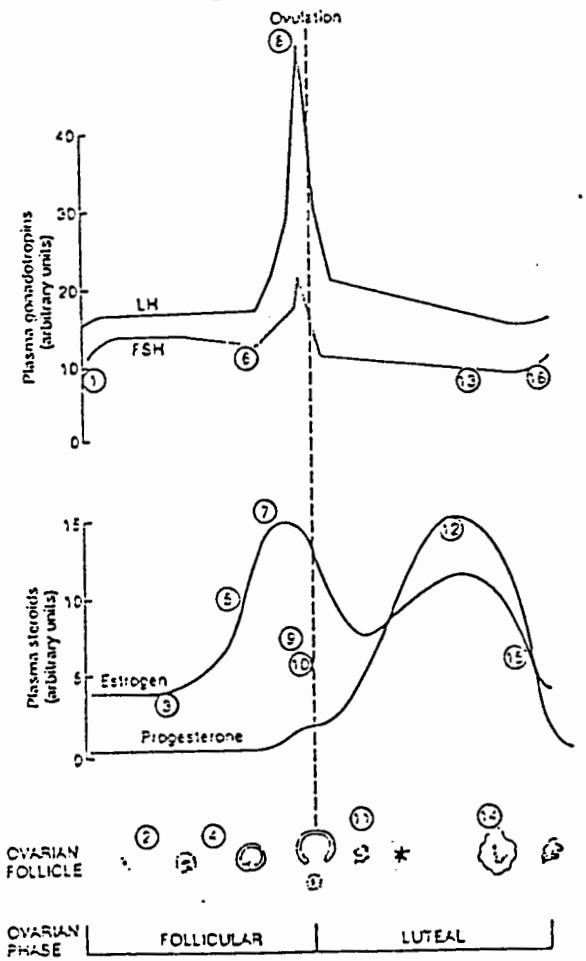


Figure 1.5. Plasma hormone concentrations and ovarian events during the menstrual cycle.

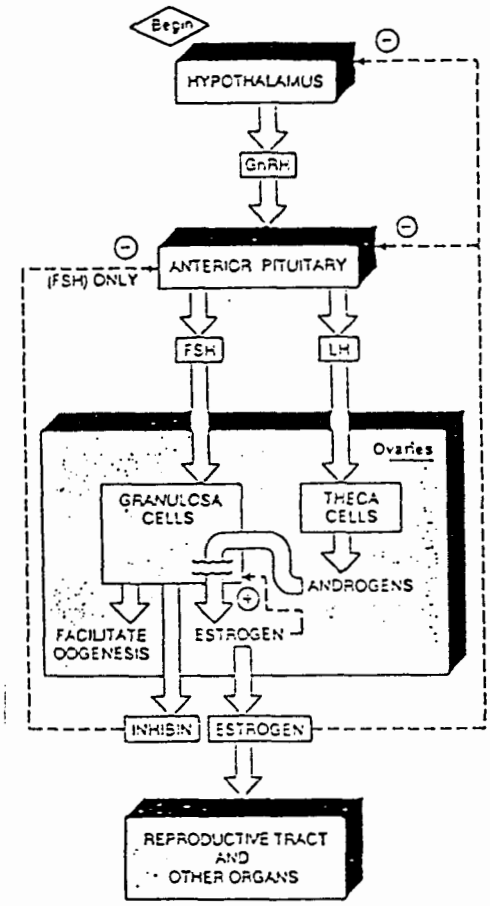


Figure 1.6. The hypothalamic-pituitary ovarian axis. GnRH = gonadotropin releasing hormone; FSH = follicle stimulating hormone; LH = luteinizing hormone.

Cycle length, the follicular phase, occurrence of ovulation and the luteal phase are parameters used to characterize the menstrual cycle as eumenorrheic, oligomenorrheic or amenorrheic (Greene 1993). Women who have a cycle length within the normal range of 21-45 days are classified as eumenorrheic while oligomenorrhea refers to too few cycles, 45-90 days duration (Greene 1993). The absence of menses for three months or longer is identified as amenorrhea (Greene 1993). Menstrual irregularities appear to be the result of changes in hormone concentrations and secretory patterns (Greene 1993). Hormonal alterations which may be involved in exercise related menstrual dysfunction are listed below (Greene 1993).

Factors predisposing female athletes to menstrual irregularities

- prior menstrual dysfunction or delayed menarche
- stress-physical and psychological
- miles run per week-high energy output
- weight loss
- undernutrition and vegetarian diets
- low body fat
- nulliparity

Oligomenorrhea and amenorrhea are found in female athletes with the severity of the disturbances dependent on intensity of training (Arena et al. 1995). Feicht et al. (1978) found a positive correlation between number of miles run per week and incidence of amenorrhea.

Menarche, defined as the onset of menses, is dependent on the hypothalamic pituitary ovarian axis (Greene 1993). Physical exercise at a young age is believed to be a causative factor in delayed menarche (Frisch et al. 1981; Malina et al. 1973; Warren 1980).

Little work has been done in rats to determine the effects of exercise on the estrous cycle (Erich et al. 1985; Pellerin-Massicotte et al. 1987; Carlberg and Fregly 1985; Sylvester et al. 1989). Vaginal opening as an indicator of sexual maturation is delayed by intense training (Erich et al. 1985; Pellerin-Massicotte et al. 1987). In an attempt to establish an animal model for studying exercise induced amenorrhea, Carlberg and Fregly exercised female Sprague Dawley rats for ten weeks at a work rate of 16m/min., 60m/day for 5 days per week (Carlberg and Fregly 1985). Cycle length was increased from 4.31 to 5.48 days in exercised rats). Rats that were run on treadmills 1 or 3 times per week had a significantly longer cycle than sedentary rats and those run 5 or 7 days per week had

significantly increased cycle lengths compared to the less frequently exercised rats and sedentary rats (Sylvester et al. 1989). Increasing frequency of training appears to increase cycle length (Sylvester et al. 1989).

1.15 Exercise and hormones

Exercise affects the levels of both reproductive and non-reproductive hormones. Blood levels of hormones depend on a balance between production, metabolism and clearance rates (Arena et al. 1995). In response to acute exercise, estradiol has been shown to either increase (Bonen et al. 1983; Jurkowski et al. 1978) or not change (Kindermann 1984; Loucks & Horvath 1984) during exercise. Others have shown that estradiol increases immediately post exercise and during recovery (Wallace 1981). Because alterations in steroid levels are delayed, changes may not occur until the end of prolonged exercise or during recovery (Shangold 1984). However the increases in estradiol and progesterone are related to the intensity of the exercise (Jurkowski et al. 1978). Heavy exercise causes significant increases in ovarian hormones in untrained subjects (Bonen et al. 1983) whereas no difference is observed in trained athletes exercising at the same absolute workload (Bonen et al. 1979; Jurkowski et al. 1978; Keizer et al. 1980; Keizer et al. 1981). Trained individuals have lower resting estradiol levels (Boyden et al. 1983). Strenuous exercise can bring on exercise induced amenorrhea which is distinct from hypothalamic amenorrhea. In the former there is no involvement of the pituitary control system which regulates menstrual fluctuations. There are conflicting reports as to whether basal estradiol levels are lower in amenorrheic runners compared to regularly menstruating runners and nonrunners. Lower levels of estradiol and sex hormone binding globulins (to which estradiol and testosterone bind) were reported among amenorrheic runners compared to eumenorrheic runners or nonrunners (Baker et al. 1981; Snead et al. 1992; Loucks and Horvath 1984). Schwartz et al. (1981) found no difference among the three groups. However the ratio of

estrone/estradiol is significantly higher in all amenorrheic subjects. These runners also display higher lutenizing hormone and dehydroepiandrosterone sulphate and lower thyroid stimulating hormone levels. This suggests that exercise associated amenorrhea is distinct from hypothalamic amenorrhea (Terblanche 1989). Contradicting studies show amenorrheic women have decreased plasma follicle stimulating hormone, luteinizing hormone concentrations at rest and there is no response of prolactin after exercise when compared to eumenorrheic runners (Loucks and Horvath 1984). Women who engage in heavy and prolonged exercise exhibit low levels of luteinizing hormones (Jones 1949; Cumming et al. 1985a; Cumming et al. 1985b; Veldhuis et al. 1985; Boyden et al. 1982).

Prolactin levels rise during exercise (Hale et al. 1983; Mayer et al. 1980; Shangold et al. 1981). Because prolactin has a short half life (10 min.) levels are back down to baseline within 45 minutes of cessation of exercise (Brisson et al. 1980). Exercise induced prolactin release only occurs in athletes suggesting a possible role of training in conditioning the hypothalamopituitary exercise induced secretion (Rolandi 1985). Increased prolactin levels occur as a consequence of repeated exercise bouts (Bullen et al. 1984; Chang et al. 1986). This may be an added causative factor for the onset of menstrual problems in addition to changes in production, metabolism and clearance of reproductive hormones (Shangold et al. 1979; Evans et al. 1982; Boyden et al. 1982). DeMeirleir et al. (1985) showed that the increase in prolactin levels during and after exercise are due to pituitary secretion rather than decreased elimination. Enhanced prolactin secretion induced by exercise may prevent ovarian aromatisation of androgens to estrogens (Keizer et al. 1987). Prolactin responses are blunted in amenorrheic runners as compared to eumenorrheic (De Souza et al. 1991).

1.16 Exercise and breast cancer

Epidemiologic and laboratory data indicate that certain patterns of physical activity may alter the risk for cancer (Kohl et al. 1988; Shepard 1986,1990). Epidemiological studies on the association between physical activity and mammary tumorigenesis have found lower, greater and no effects on lifetime occurrence of breast cancer (Paffenberger et al. 1987; Frisch 1987). Frisch et al. (1987) found that there is a lower incidence of breast cancer among former college athletes compared to there sedentary counterparts. Similar results were reported for the prevalence of benign lesions (Wyshak et al. 1986). Paffenberger et al. (1987) found no relationship between exercise during the college years and breast cancer incidence. There are inherent problems with epidemiological studies of this nature. Results are based primarily on questionnaires answered by subjects which requires them to remember what they did 20 years ago. The data from the Paffenberger study was originally collected for a hypertensive-atherosclerotic disease study. Therefore some controls were missing. As Cohen (1991) states in a review on the topic, the greatest weakness of epidemiological studies to date has been the assessment of activity status.

A number of animal studies (rats and mice) have examined the effects of exercise on 7,12-dimethylbenz(a)anthracene (DMBA) or nitrosomethylurea (NMU) induced mammary tumors. The majority of the investigations have studied the effects of exercise on the promotion phase of mammary tumorigenesis. Primarily, two types of exercise have been utilized, voluntary access to a running wheel and forced treadmill running. Voluntary exercise has been consistently shown to decrease tumor incidence. Cohen et al. (1988,1991) found that allowing rats free access to activity wheels after NMU administration decreased tumor yield and increased tumor latency. These results were supported in a study by Benjamin et al. (1988). The studies utilizing forced treadmill

running have reported conflicting results. Initial studies by Thompson et al. (1988,1989) in which a very low intensity exercise regime was utilized revealed that when exercise was initiated post-DMBA administration, there was a higher tumor incidence and tumor number and decreased tumor latency regardless of whether the rats were fed a high fat or low fat diet. In a more recent paper, Thompson (1992) has found that increasing the duration from 15 to 30 min. per training session delayed tumor appearance in rats injected with NMU. Lane et al. (1991) found that exercising mice during the promotion phase of DMBA induced mammary tumorigenesis lowered tumor incidence. These studies provide evidence to suggest that, in rats and mice, exercise can alter the incidence and latency of mammary tumors induced by carcinogens.

In animal models, the effects of exercise prior to carcinogen administration on subsequent tumor development has received relatively little attention. Yedinak (1988) found that exercise prior to DMBA administration and during the initiation phase decreased tumor incidence with a low fat diet and increased incidence with a high fat diet. A follow up study looked at the relationship between exercise and mammary tumorigenesis using two doses of DMBA (Layman et al. 1990). They found that a lower dose plus exercise led to a decreased incidence and increased latency, whereas the higher dose plus exercise had the opposite effects.

There are inherent problems in comparing the results from experimental animal studies. They are the types of exercise, intensity and duration of exercise, type of carcinogen (DMBA is host activated, NMU is direct acting) and the length of the experiment.

1.17 Insulin-like growth factor 1

Insulin-like growth factors (IGFs), classified as type I and type II are polypeptides with structural homology to insulin. IGFs can elicit two types of biological responses: 1) rapid metabolic effects (insulin like); and/or 2) slower growth promoting effects (mitogenic) (Krywicki et al. 1992). IGFs allow the cell to progress from G1 to S phase of the cell cycle (Krywicki et al. 1992). IGF-I exerts its effects by binding to the IGF-I receptor and activating its tyrosine protein kinase (Rosen et al. 1991). IGF-I mediates the effect of growth hormone, which is produced in the pituitary gland. Growth hormone induces IGF-I production in a number of tissues (Lowe 1991; D'Ecrole et al. 1984; Walker et al. 1991). Because growth hormone is involved in mammary gland development, a natural progression is to study the presence of IGF-I in mammary tissue. There are several lines of evidence which suggest that IGF-I is involved in the growth and differentiation of the mammary gland including: 1) the stimulation of IGF-I mRNA by growth hormone in rat mammary glands (Kleinberg et al. 1990); 2) presence of IGF-I mRNA in rat adult tissue and human mammary stromal tissue (Murphy et al. 1987; Yee et al. 1989) 3) mammary epithelial cells produce and secrete IGF-I binding proteins (IGFBP) which may play a role in regulation of action of IGF-I (McGrath et al. 1991; Campbell et al. 1991) 4) primary cultures from animals are stimulated by physiologic concentrations of IGF-I (Cullen et al. 1992).

IGF-I is mitogenic for some breast cancer cell lines in tissue culture (Furlanetto and DiCarlo 1984; Karey and Sirbasku 1988; Cullen et al. 1990). There is debate as to whether or not IGF-I is expressed in mammary tumors. By northern blot analysis of breast cancer cell lines, IGF-I mRNA was detected (Huff et al. 1986). However, Subsequently it was subsequently determined using the more sensitive RNase protection assay that the mRNA

are not authentic IGF-I transcripts but some related substance that also crossreacts in radioimmuno assays (Yee et al. 1989). In both normal and tumor breast tissue, the RNase protection assay reveals authentic mRNA transcripts (Yee et al. 1989). The IGF-I mRNA is expressed in stromal fibroblasts as indicated by *in situ* hybridization (Yee et al. 1989). This suggests IGF-I may act through a paracrine mechanism. In tumor tissue, IGF-I mRNA expression is detected in the normal area of the specimen and not in stroma adjacent to tumor cells (Yee et al. 1989). IGF-II mRNA shows similar results to IGF-I mRNA (Rosen 1991). Again, in normal and malignant tissue it is expressed in stromal cells (Rosen 1991). However, in breast tumor tissue it is expressed in adjacent stromal cells. Also some malignant epithelial cells express IGF-II mRNA (Rosen 1991). Therefore IGF-I may act in endocrine (due to IGF-I in serum) or paracrine fashion and IGF-II in paracrine or autocrine fashion.

1.18 Insulin-like growth factor 1 receptor

The insulin-like growth factor I receptor is a heterotetramer with structural homology to the insulin receptor (Krywicki et al. 1992). It is synthesized as a single peptide (180 kDa) which is subsequently glycosylated and cleaved to an α and β chain (Krywicki et al. 1992). The α chains (apparent molecular weight = 135 kDa) are extracellular and covalently bound by a disulfide bridge to the predominantly intracellular β chains (apparent 90 kDa) in β–α–α–β configuration (figure 1.7) (Krywicki et al. 1992).

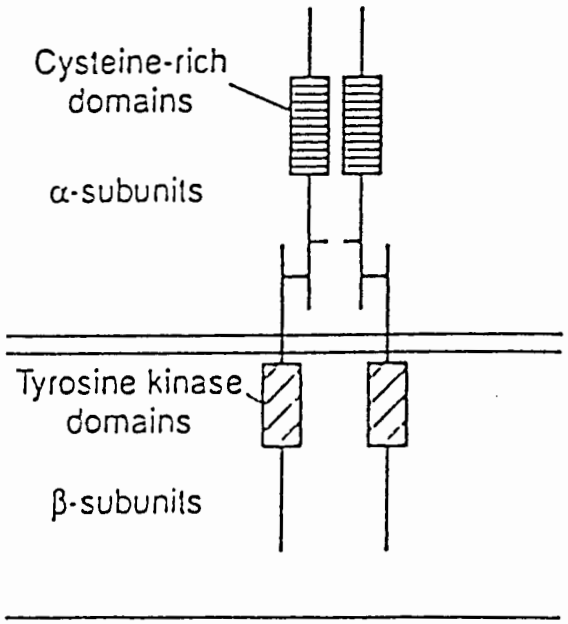


Figure 1.7. The IGF-I receptor.

The α chains contain ligand binding domains whereas β chains span the cellular membrane and possess tyrosine kinase activity on the cytosolic side of the membrane (Ullrich et al. 1986; Steele-Perkins et al. 1988). Insulin-like growth factor I receptor binds IGF-I and II with roughly equal affinities while the affinity for insulin is much lower (Humbel 1990). A monoclonal antibody (αIR3) directed against the IGF-I receptor blocks the IGF-I response indicating the mitogenic signal of IGF-1 is mediated via the IGF-1 receptor (Flier et al. 1986).

The presence of IGF-I receptor in most studies to date has been detected using ligand binding assays. Most breast cancer cell lines express IGF-I receptor (Cullen et al. 1990; Furlanetto and DeCarlo 1984; DeLeon et al. 1988). IGF-I receptor concentrations are

higher in hormone dependent cell lines than hormone independent cell lines (Peyrat and Bonneterre 1992). There is a positive link between IGF-I receptor and estrogen receptor, progesterone receptor or prolactin receptor (Peyrat et al. 1988a; Peyrat and Bonneterre 1992). The correlation is stronger in postmenopausal women (Peyrat and Bonneterre 1992). IGF-I receptor is detected in normal and malignant breast tissue (Pollack et al. 1987; Peyrat et al. 1988b; Pekonen et al. 1988; Foekens et al. 1989a). The expression of the IGF-I receptor is associated with good prognosis, estrogen receptor positive cancers (Foekens et al. 1989b).

IGF-I receptor mRNA is present in all breast cancer cell lines as tested by RNA protection assay (Cullen et al. 1990). In MCF 7 cells which are hormone dependent, IGF-I receptor mRNA levels are stimulated by estradiol (Stewart et al. 1990; Freiss et al. 1990). Breast cancer biopsies reveal similar results to cell lines, ie. the presence of IGF-I receptor as detected by a binding assay (Peyrat et al. 1989; Cullen et al. 1990; Peyrat et al. 1990).

In terms of Specificity, IGF-I is slightly more potent than IGF-II (2X) in competing for IGF-I binding and 100 fold more potent than insulin (Peyrat and Bonneterre 1992). IGF-I and II receptors are ubiquitous in human breast cancer, however, evidence suggests that some of the biological effects of IGF-II are mediated through type I IGF receptor (Keiss et al. 1987; Roth 1988). α IR-3 mAB which blocks the type I receptor abolishes mitogenic effects of both IGF-I and II in breast cancer cell lines and tumor biopsies (Cullen et al. 1990; Osborne et al. 1989).

Overall survival (OS) and relapse free survival (RFS) correlates with IGF-I receptor presence in tumors (Bonneterre et al. 1990). There is no difference between node positive or negative patients or tumor diameter (Peyrat and Bonneterre 1992). IGF receptor binding has been shown to be higher in tumor tissues than adjacent normal tissue suggesting the

IGF-I receptor expression is associated with malignant transformation of breast epithelial cells (Pekonen et al. 1988).

1.19 Estrogen

Estrogens are a class of steroid hormones produced by the ovaries in premenopausal women whereas conversion of androgen to estrogen in adipose tissue is the primary source in postmenopausal women (Miller 1990). Estrogen is involved in the growth of both normal and neoplastic breast tissue. The role of estrogen in normal mammary gland development was reviewed earlier. The establishment of estrogen's role in breast cancer comes from the following lines of evidence: 1) estrogen acts as promoting agent for rodent mammary tumors (Henderson et al. 1988; Vessey 1989); 2) protective effect of ovariectomy (Miller 1990; Henderson et al. 1988; Vessey 1989); 3) occurrence of breast cancer is rare in males (Miller 1990); 4) estrogen's mitogenic effects on breast cancer cell lines (Dickson and Lippman 1988; Darbre and King 1988); and 5) successful treatment of some breast cancers with antiestrogens (Santen et al. 1990; Miller 1990). There are a number of mechanisms through which estrogen may modulate breast tumor growth which are outlined below in Figure 1.8 (Miller 1990). The hormone must bind to the estrogen receptor for estrogen to have direct effects on tumor growth.

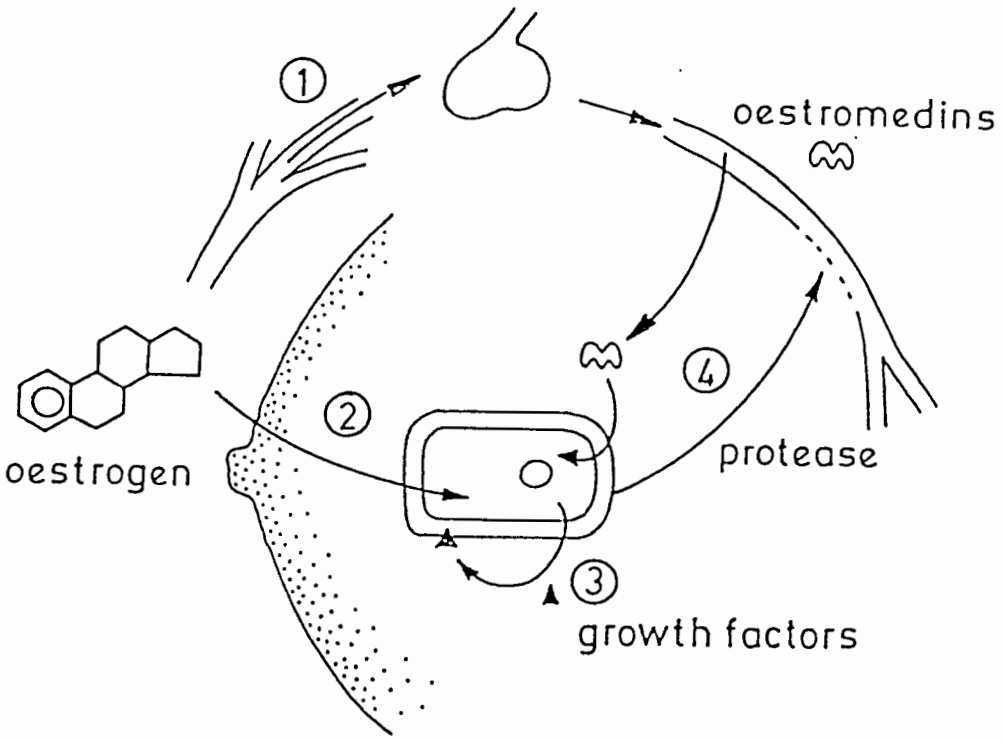


Figure 1.8. Mechanisms of oestrogen action in promoting the growth and metastasis of tumor cells.

1) oestrogens cause the release of oestromedins from the pituitary which stimulate tumor cells.

2) oestrogen has direct effects on tumor cell growth.

3) oestrogens stimulate the release of mitogenic growth factors from tumor cells.

4) oestrogen stimulates the release of proteases from tumor cells.

1.20 Estrogen receptor

The estrogen receptor is a member of the nuclear hormone superfamily (Parker et al. 1993). It has an apparent molecular weight of 65 kDa. The receptor acts as a ligand dependent transcription factor (Baniahmad et al. 1994). The estrogen receptor is located in the cell nucleus in the inactive form (Parker et al. 1993). When estrogen binds to the receptor, a conformational change occurs, the receptor forms homodimers and binds to DNA (Parker et al. 1993; Baniahmad et al. 1994). The receptor has at least three functions: ligand binding, protein dimerization and transcriptional activation (Parker et al. 1993). The structure of the receptor can be divided into three parts, the amino terminus (containing the A/B region), the DNA binding domain (C region) and the C terminus (containing the D/E/F region) (Baniahmad et al. 1994). The functional domains are outlined in figure 1.9 (Baniahmad et al. 1994).

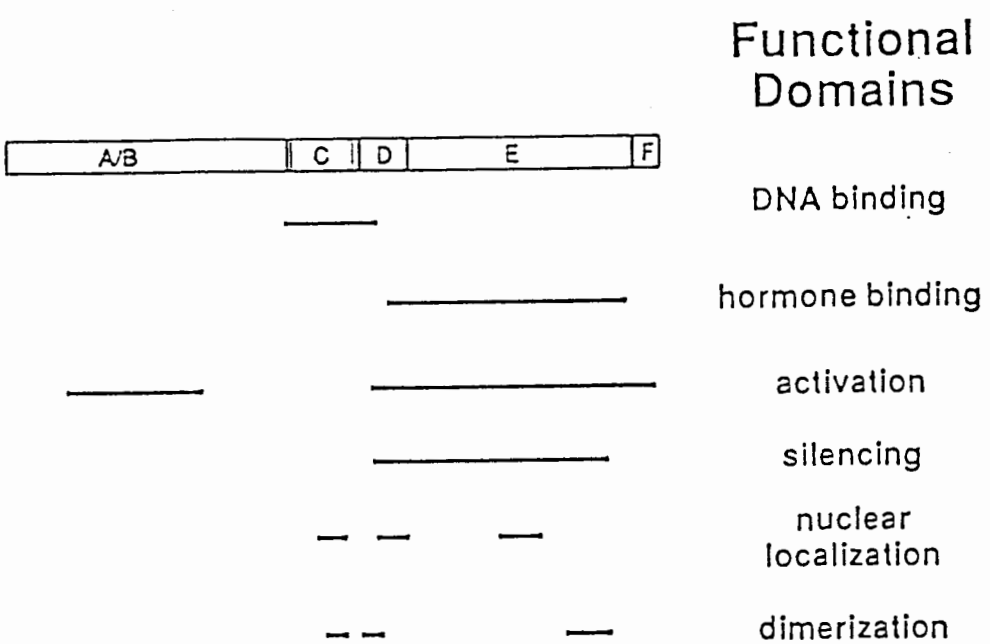


Figure 1.9. The functional domains of the estrogen receptor.

Estrogen receptors have been identified in both normal and malignant breast tissue (Reviewed by Auchus and Fuqua 1994). Estrogen receptor status, determined by ligand binding assays, is used to ascertain the responsiveness to hormone therapy. Those tumors that are estrogen receptor positive respond to this therapy and have a more favorable prognosis (Auchus and Fuqua 1994).

1.21 Rationale

Breast cancer is the leading cause of death among North American women. Although the exact etiologic agents of breast cancer are not known, many epidemiological and laboratory studies have focused on lifestyle changes that may reduce the risk of breast cancer. As cancer is not a static disease, but rather a multistep process (initiation to promotion to progression), there are a number of steps where intervention is possible. Epidemiological studies have shown that exercise at an early age lowers the prevalence of breast cancer. Although laboratory studies have assessed the effects of exercise during the promotion phase of tumorigenesis, none have addressed the question of exercise prior to initiation and its effects on susceptibility of the mammary gland to carcinogenic attack. By using an animal model to answer this question variables such as diet, exercise intensity/duration and genetics can be controlled. As exercise is known to alter a number of factors which are thought to modify the carcinogenic process including endocrine responses and metabolic activities, potential mechanisms affecting tumor development will be studied.

1.22 Justification of model

Female Sprague Dawley rats were chosen because 1) they are relatively complaint in terms of running on treadmills and 2) they are susceptible to NMU induced mammary

tumorigenesis. NMU shows organ site specificity in terms of induction of mammary tumors. The induced tumors are primarily adenocarcinomas and are locally invasive. Like human breast tumors, NMU tumors in rats are estrogen dependent. Finally, the NMU model was chosen for these experiments because it is direct acting, requiring no host activation. The purpose of this work was to assess the effects of exercise on the mammary gland itself and subsequent tumor development rather than the metabolism of a carcinogen.

Chapter II Experimental strategy

The purpose of this thesis was to assess the effects of exercise in young female Sprague Dawley rats on mammary gland development; carcinogen distribution, binding and damage; and subsequent mammary tumor development. Exercise is known to alter the levels of hormones involved in mammary gland development and epithelial cell differentiation, two factors involved in the susceptibility of the mammary gland to carcinogenic attack. Chapter three presents experiments which determined if exercise at different intensities and/or durations alters mammary gland development and epithelial cell proliferation. In order for a carcinogen to be effective, it must first reach the target cell and cause DNA damage. Experiments presented in chapter four assess the distribution and binding properties of NMU at three different doses. The effects of exercise on DNA damage/repair was determined using the nick translation assay. The experiments in chapter five assess the effects of exercise prior to NMU administration on subsequent mammary tumorigenesis utilizing two different doses of NMU, 50 mg NMU/kg and 37.5 mg NMU/kg. The final experiments in chapter six determine the estrogen receptor and IGF-I receptor content in tumors from sedentary and exercised rats and examine possible relationships between receptor content and tumor parameters such as growth rate, latency and weight.

It was hypothesized that exercise prior to carcinogen administration would decrease tumor incidence and increase latency period. This effect would be more pronounced with the lower dose. The factors responsible for this effect of exercise were hypothesized to be twofold. First, through the alteration of hormone levels, exercise would enhance mammary gland development and decrease proliferation. Secondly, exercise would alter the distribution of NMU, clear it from the body faster and decrease DNA damage.

Chapter III Effects of exercise during puberty on mammary gland development and mammary epithelial cell proliferation

3.1 Introduction

Breast cancer is a physically and psychologically debilitating disease. The ultimate goal in breast cancer research is to find a way of preventing the disease. One of the potential risk factors for breast cancer is thought to be the cumulative exposure to estrogen (Henderson et al. 1985; Henderson et al. 1988). Cumulative exposure takes into account age at menarche, number of ovulatory cycles, age of first pregnancy, and age of menopause. Strenuous exercise is thought to delay menarche and increase the number of anovulatory cycles reducing the cumulative exposure to estrogen (Malina et al. 1978; Warren 1980). In addition, Bernstein et al. (1987) have proposed that moderate intensity exercise during adolescence reduces the risk of breast cancer, as they have observed increased menstrual cycle length patterns and reduction in the number of ovulatory cycles with moderate intensity exercise training. It has been suggested by Hoffman-Goetz and Husted (1994) that exercise could effect estrogen levels and consequently breast cancer either directly by altering ovulatory cycles or indirectly through body composition. Aromatase, an enzyme found in fat deposits, is responsible for converting androgens to estrogen. Abdominal obesity is correlated with increased risk of breast cancer (Schapira et al. 1990). These findings suggest that changes in circulating hormone levels due to exercise either directly or indirectly alter the mammary gland in such a way as to provide a protective effect against breast cancer. To our knowledge no one has determined the effects of exercise directly on the normal rat mammary gland development and/or epithelial cell proliferation.

Susceptibility of the mammary gland to carcinogenic attack is partially dependent on the developmental stage of the mammary gland (Russo et al. 1982). The more differentiated the gland is, the lower the proliferation rate is, giving more time for DNA repair which results in lower susceptibility. In rats, energy restriction delays the onset of puberty characterized by vaginal opening (Arts et al. 1992 ; Park et al. 1994; Lok et al. 1990). Mammary gland development is delayed in energy restricted rats and proliferation rates in TEBs decreases (Arts et al. 1992). The effects of exercise on mammary gland development are currently not known. However, exercise is known to affect caloric balance and changes in diet have been demonstrated to affect mammary gland development (Welsch and O'Connor 1989). Exercise is thought to delay menarche and affect menstrual cycles in both humans and rats (Frisch et al. 1981; Bernstein et al. 1987; Chatterton et al. 1990; Carlberg and Fregly 1985). It has been suggested that exercise alters the endocrine levels of prolactin, estrogen, progesterone and growth hormone, which consequently affect mammary gland development (Kohl et al. 1988; Frisch et al. 1985; Bernstein et al. 1987). Albanes and Winick (1988) state that cancer risk is proportional to the number of proliferating cells and this number is dependent upon the number of cells in the tissue and the rate of cell division. We therefore hypothesized that chronic physical activity prior to carcinogen exposure may serve to alter normal mammary gland development in such a way as to affect tumor initiation and subsequent tumorigenesis.

The purpose of this study was to assess the effects of exercise during peripuberty on mammary gland development and mammary gland epithelial cell proliferation in Sprague Dawley rats. In order to assess the effects of exercise, the rats were exercised from twenty one to fifty days of age for two reasons: 1) the mammary gland develops allometrically in the rat from about twenty one days of age to sixty days of age after which time it remains relatively static until pregnancy occurs, and 2) highest multiplicity of DMBA and NMU

induced mammary carcinomas occurs when the rats are inoculated at around fifty days of age, a time when the number of undifferentiated terminal end bud (TEB) structures and the rate of DNA synthesis is at its highest in the rat (Russo et al. 1982).

3.2 Methods

Two separate experiments were conducted to determine the effects of exercise on mammary gland development and epithelial cell proliferation. The first experiment involved two groups: sedentary (S1) and exercise (moderate intensity, short duration exercise) (MS). The second experiment involved four groups: one sedentary (S2) and three exercise groups; 1) moderate intensity, long duration (ML); 2) high intensity, short duration (HS); and 3) high intensity, long duration (HL).

3.2.1 Animals

Twenty one day old female Sprague-Dawley rats were obtained from Charles River Labs. Upon arrival, the rats were randomly divided in each experiment into sedentary or exercised groups (n=10 per group). In experiment 1, two rats from each group (S1 and MS) were lost over the course of the experiment. The rats were maintained on a twelve hour light dark cycle (lights on 7 p.m./ lights off 7 a.m.) and fed laboratory rodent diet 5001 ad libitum (Ralston Purina). The rats were housed in groups of five. All rats were weighed once a week for the duration of the study.

3.2.2 Exercise protocol

The exercised rats were exercised in a separate room five times per week during the dark cycle on a progressive treadmill training as outlined in table 3.1. The treadmills were

equipped with airjets at the back in order to keep the rats moving. Electrical shock was not used in the protocol. The rats were exercised for four weeks from 21 days of age to 50 days of age.

Days	final speed	final duration	incline
22-28	MS-12 m/min.	60 min.	15%
	ML- 13 m/min.	90 min.	
	HS-14 m/min.	60 min.	
	HL-14 m/min.	90 min.	
29-35	MS-14 m/min	60 min.	15%
	ML-16.5 m/min.	105 min.	
	HS-18 m/min.	60 min.	
	HL-18 m/min.	105 min.	
35-42	MS- 16 m/min	60 min.	15%
	ML- 18 m/min.	105 min.	
	HS-20 m/min.	60 min.	
	HL-20 m/min.	105 min.	
43-49	MS- 18m/min	60 min.	15%
	ML- 18 m/min.	120 min.	
	HS-20 m/min.	60 min.	
	HL-20 m/min.	120 min.	

Table 3.1. Exercise training protocol. Rats were exercised from twenty one days of age to fifty days of age on a motorized treadmill. MS= moderate intensity, short duration; ML=moderate intensity, long duration; HS=high intensity, short duration; HL=high intensity, long duration.

3.2.3 Tissue sampling

The rats were sacrificed at 50 days of age (24 hours after the last bout of exercise) via CO_2 asphyxiation. Mammary glands four and five were removed and fixed in methacarn for determining BrdU labeling index, which is used as an indice of proliferation rate. Mammary gland six was removed and processed for whole mount evaluation to assess developmental stage. For the exercise groups ML, HS, HL and the sedentary group S2, blood was collected via cardiac puncture and centrifuged at 600 x g for fifteen minutes. The serum was removed and stored at -20^{0}C for the assessment of plasma prolactin levels. Soleus muscles were removed and stored at -20^{0}C for the assessment of citrate synthase activity.

3.2.4 Bromodeoxyuridine (BrdU) Labeling Index

At 46 days of age the rats were injected twice daily (7:30 a.m. and 7:30 p.m.) ip. for four days with 0.15 mls of 20 mg/ml BrdU in 0.9% NaCl (pH 4.0) for a total of 58.6 µmol BrdU (ie. 8 injections per rat) immediately prior to termination. The exercised rats maintained their training during the four days of labeling. The four day (continuous labeling) approach is used because the labeling index (LI) is low in the mammary gland and it is affected by the stage of the estrous cycle, which is problematic in terms of sensitivity and sequencing a kill for an estrous cycle specific determination of LI. After fixation in methacarn (24 hours), the tissues were processed in the following sequence: a) absolute methanol-3hrs b) absolute methanol-3hrs c) methyl benzoate-3hrs d) methyl benzoate-3hrs e) xylene-1hrs f) paraffin-1hrs g) paraffin-1hrs h) paraffin-4hrs.

The tissue was embedded in paraffin blocks and 5 micron sections onto poly-L-lysine coated slides. Heat immobilized sections (60^{o}C for 30 min.) were deparaffinized in xylene (3 x 5 min), immersed in descending grades of ethanol (100% ethanol 2 x 5 min.; 95% ethanol 1 x 5 min.; 80% ethanol 1 x 5 min.), rinsed in deionized water and subjected to mild acid hydrolysis with 2N HCl for 90 min. The slides were neutralized with 0.1M sodium

borate (1 x 5 min.). After rinsing with deionized water, endogenous peroxidase activity was blocked by immersing slides in 1.5% hydrogen peroxide (1 x 5 min.). After rinsing with phosphate buffered saline (PBS) (3 x 5 min.), mouse Anti-BrdU (Becton Dickinson) (1:40) was applied for one hour. Slides were washed with PBS (3 x 5 min.) and then biotinylated rabbit anti-mouse (DAKO) (1:200) was applied for 30 min. After another wash with PBS (3 x 5 min.), conjugated streptavidin horse radish peroxidase (DAKO) (1:1000) was applied for thirty minutes. A wash in PBS (3 x 5 min.) was followed by a 10 min. incubation with diaminobenzadine (Sigma) and a rinse in deionized water. The slides were stained with hematoxylin (1 x 1min.), then dehydrated through a series of ethanols and xylene (95% ethanol 1 x 1 min.; 100% ethanol 2 x 1 min.; xylene 3 x 1 min.). The tissue was mounted with permount and scored according to a procedure adapted from Meyer et al. (1977). For each animal, sections of the entire mammary gland were screened to assess random distribution of proliferative cells. Five hundred consecutive ductal nuclei were counted and the number that stained positive noted. Similarly, 500 consecutive lobuloalveolar nuclei were counted and the number of positive nuclei within them noted.

3.2.5 Whole Mounts

Mammary gland six was removed and spread on a glass slide. The tissue was fixed in methacarn for twenty four hours. The fixative was removed and the slides were processed through a series of ethanol (100%, 95%, 70%) immersions of one hour each. This was followed by thirty minutes in distilled water. The tissue was stained with Alum Carmine overnight with distilled water being used for destaining. The slides were processed through the following series: two changes 70% ethanol, one hour each; two changes 95% ethanol, one hour each; two changes 100% ethanol, one hour each. The slides were then left in xylene overnight. The xylene was replaced with methyl salicylate and left for 2-3 days after which the methyl salicylate was replaced with xylene and left overnight. The slides were

then mounted with permount. The scoring for development was adopted from Welsch and O'Connor (1989): score 1= few ducts, few or no end ducts; score 2= moderate duct growth, moderate no. of end ducts; score 3= numerous ducts and branches, many end ducts; score 4= numerous ducts and branches, minimal lobuloalveolar growth; score 5= numerous ducts and branches, moderate lobuloalveolar growth; score 6= numerous ducts and branches, dense lobuloalveolar growth as in late pregnancy.

3.2.6 Prolactin assay

The rat prolactin EIA kit from Diagnostic Products Corporation (DPC) was used to assess basal prolactin levels in the serum of exercised and sedentary rats. Briefly, 25 µl of standards, controls and serum samples were incubated with ligand labeled murine monoclonal anti-rat prolactin antibody and horseradish peroxidase-labeled murine monoclonal anti-rat prolactin antibody (100 µl each) for two hours in a ligand-coated microplate. Rat prolactin anti-ligand(25 µl) is added to all samples and incubated for one hour. All wells are washed four times and then substrate working solution (o-phenylenediamine tablet, 25 ml rat prolactin buffered peroxide solution) is added (200 µl). The samples are incubated in the dark for thirty minutes. Stop solutions are added to all wells (50 µl and the plate is read at 492 nm. A standard curve is generated and the concentrations of the unknowns is interpolated and expressed as ng/ml.

3.2.8 Statistical analysis

Animal weights and prolactin levels were analyzed by one way analysis of variance (ANOVA). The development scores and BrdU labeling index was analyzed by the Mann

Whitney U test in experiment 1 and by the Kruskal Wallis test in experiment 2. Posthoc analysis was performed using the Tukey test for nonparametric data.

3.3 Results

In order to assess the effects of exercise on mammary gland development and epithelial cell differentiation, two sets of experiments were conducted. In the initial study, rats exercised at a moderate intensity, short duration (MS) and sedentary rats (S1) were compared (Table 3.1). To determine if intensity and/or duration had an effect on developmental scores or BrdU labeling index, a second set of experimental rats were divided into a sedentary group and three exercise groups (groups ML, HS, HL) which were run according to the protocol in Table 3.1. The gain in weight was recorded over the course of the exercise regime (Figure3. 1). There was no difference in animal weight between the exercised rats and their respective sedentary rats.

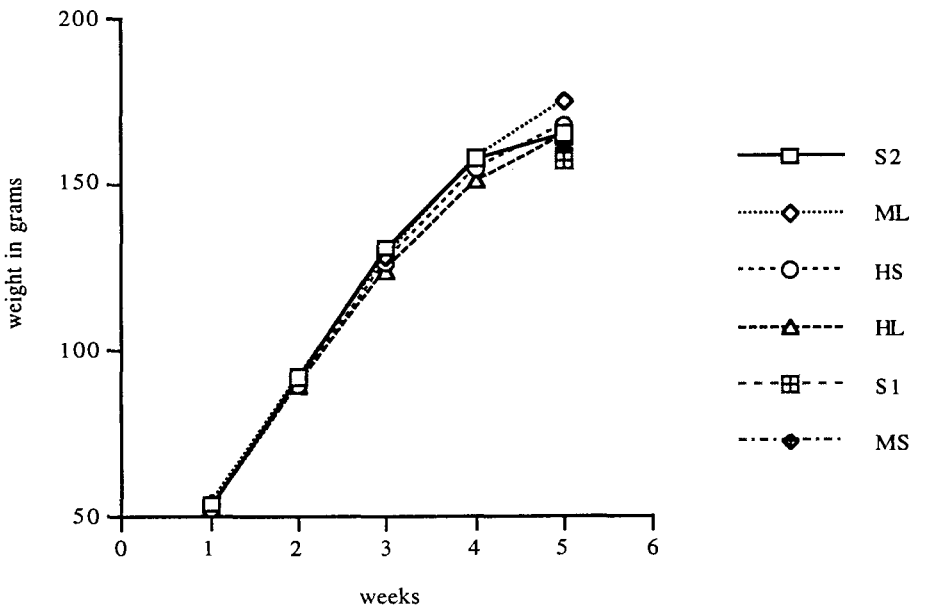


Figure 3.1. Animal weight gain over the course of the experiment in sedentary and exercised rats. The rats were exercised from twenty one to fifty days of age. There were no differences ($p>.05$). The values are expressed as means. S1= sedentary rats from experiment 1, S2= sedentary rats from experiment 2, MS= moderate intensity; short

duration; ML= moderate intensity, long duration; HS= high intensity, short duration; HL= high intensity, long duration.

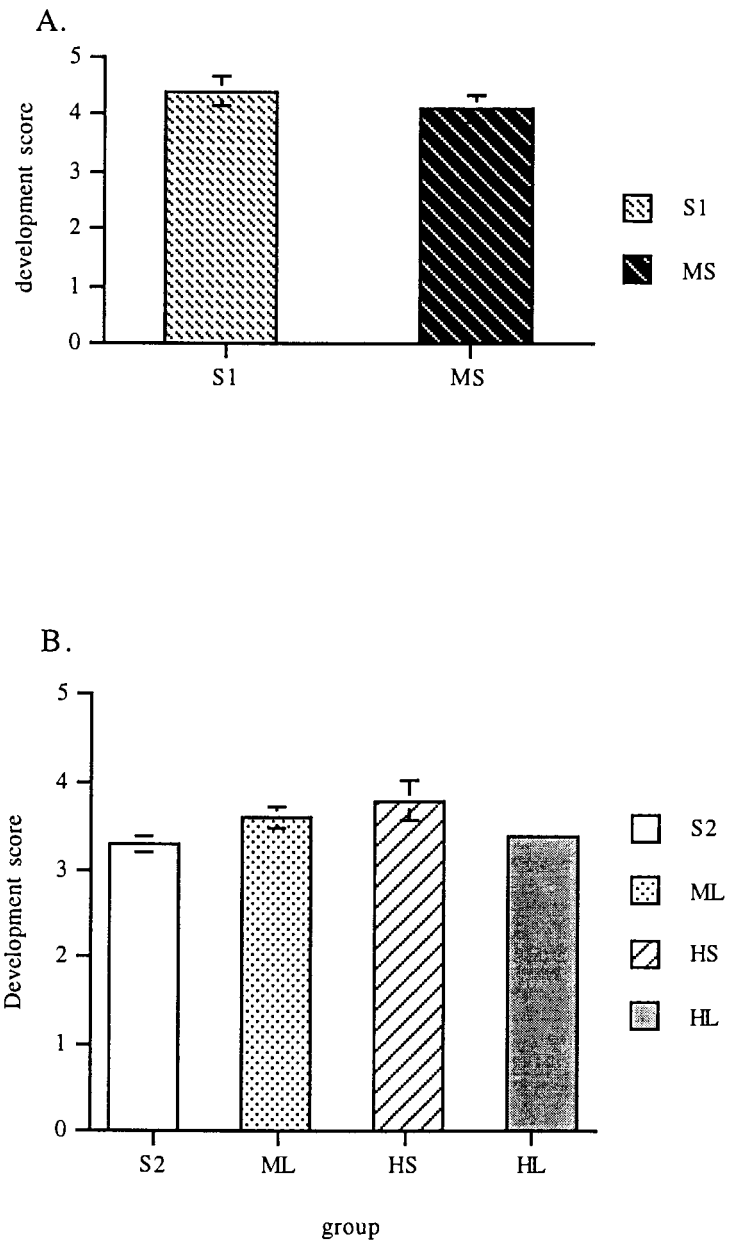


Figure 3.2A and 3.2B. Developmental scores for sedentary and exercised groups. The rats were exercised from 21 days of age to 50 days of age, then sacrificed and mammary gland six was removed for whole mount preparation. There were no differences between the sedentary and exercised groups($p>.05$). The values are expressed as means $\pm$ S.E. Refer to figure 3.1 for figure legend.

The developmental status of the mammary glands at 50 days of age was assessed by scoring whole mount preparations of mammary gland six (figure 3.2A and 3.2B). Four weeks of exercise (regardless of intensity and/or duration) did not affect the development score at the time point tested, 50 days of age.

To assess the affects of chronic exercise on basal prolactin levels, blood samples were collected twenty fours hours after the last bout of exercise. There was no difference in prolactin levels between the sedentary and exercised groups (Figure 3.3).

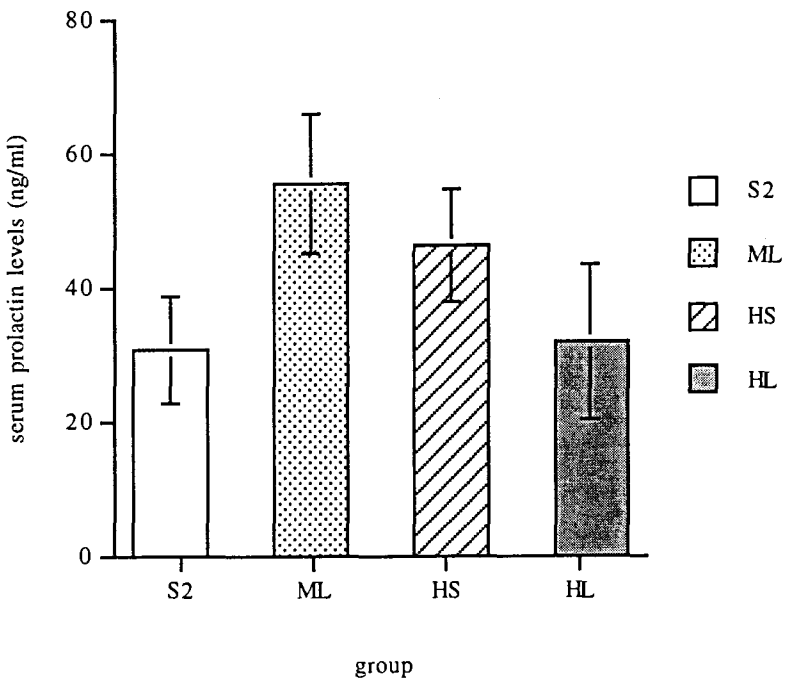
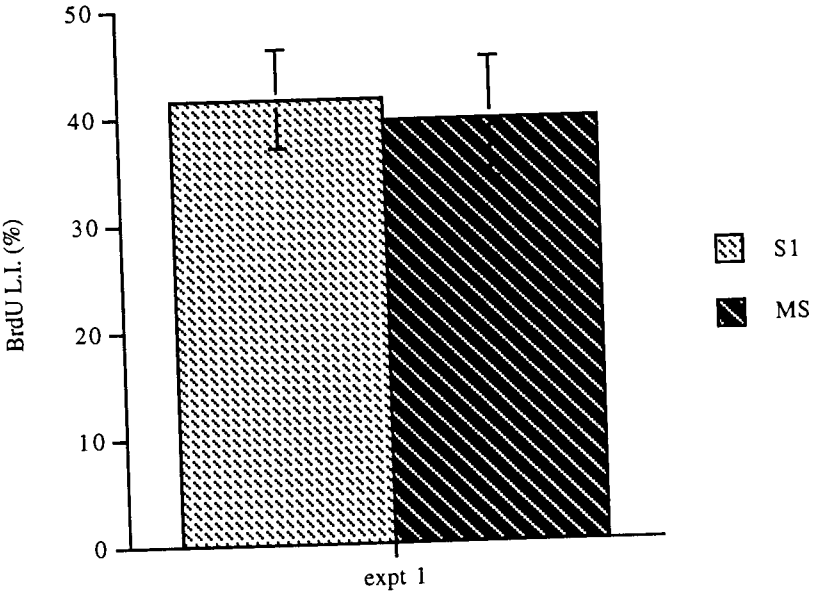
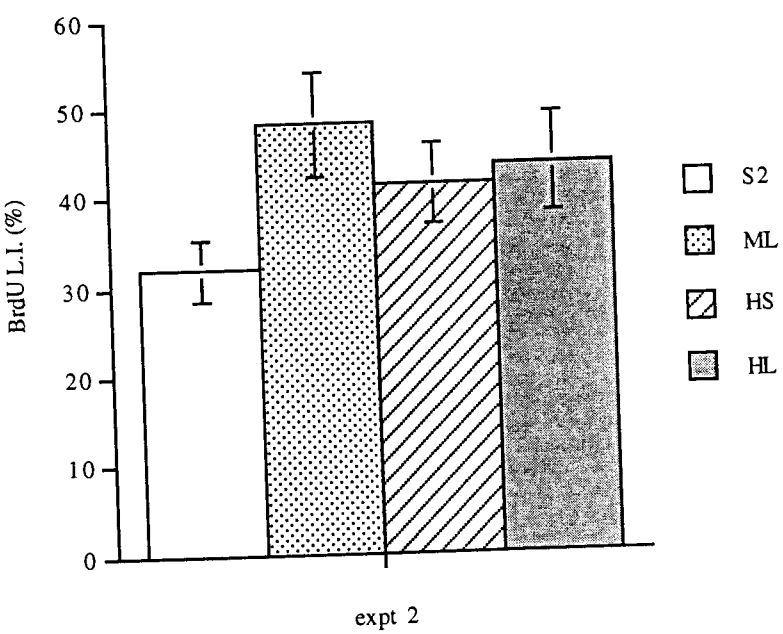


Figure 3.3. Basal serum prolactin levels. Serum samples were collected twenty four hours after the last bout of exercise. There were no differences between the sedentary and exercise groups($p>.05$). The values are expressed as means $\pm$S.E. Refer to figure 3.1 for figure legend.

The proliferation rate of mammary epithelial cells from sedentary and exercised rats was assessed by the bromodeoxyuridine labeling index (BrdU L.I.). The ductal and lobuloalveolar cells were scored separately. For the ductal cells, there was no difference in BrdU L.I. between sedentary and exercised groups (Figure 3.4A). However, the proliferation rate of the lobuloalveolar cells was significantly greater in all exercise groups compared to sedentary groups (Figure 3.4B). There was no difference between the three exercise groups.

A.


50
40
30
20
10
0
BrdU L.I. (%)
S1
MS
expt 1


60
50
40
30
20
10
0
BrdU L.I. (%)
S2
ML
HS
HL
expt 2

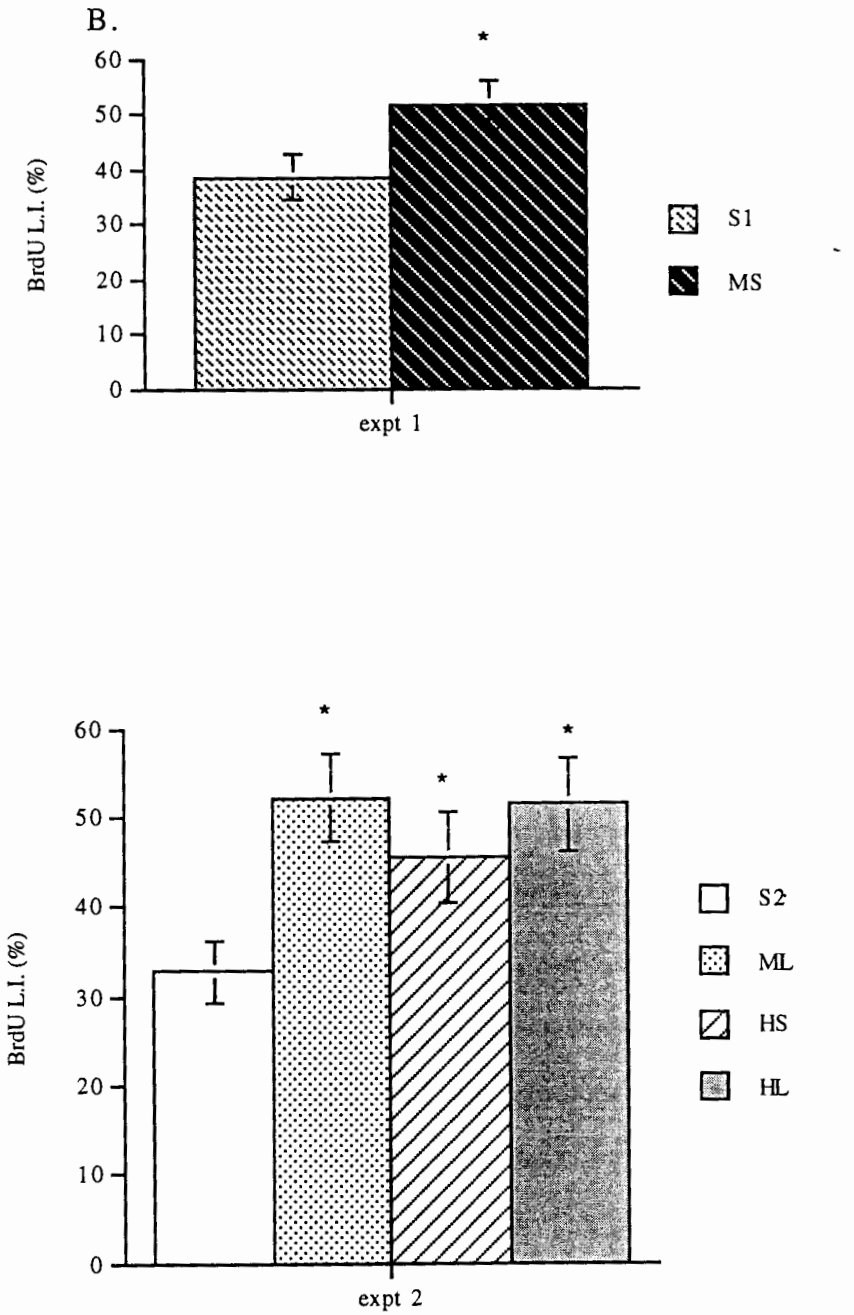


Figure 3.4. BrdU labeling index for A) ductal and B) lobuloalveolar cells from sedentary and exercised groups. The rats were exercised from 21 days of age to 50 days of age, then sacrificed and mammary glands 4 and 5 were removed. The bromodeoxyuridine labeling index (BrdU L.I.) is calculated as the percentage of proliferating cells. Ductal cells and lobuloalveolar cells were calculated separately. * The Lobuloalveolar cells from exercised rats have a significantly higher BrdU L.I. ($p<.05$). The values expressed are the means $\pm$ S.E. Refer to figure 3.1 for figure legend.

3.4 Discussion

In this study, we assessed the effects of varying exercise intensity and/or duration on mammary gland development and epithelial cell proliferation. Exercise did not alter mammary gland development or serum levels of prolactin, a mammotrophic hormone. However, exercise significantly increased the proliferation rate of lobuloalveolar cells in the mammary gland. This was contrary to what we expected. Our hypothesis was that exercise would lower the proliferation rate, and 2) increase the degree of differentiation of mammary gland cells with an increased cell cycle.

Mammary gland development, assessed at 50 days of age, was the same for sedentary and exercised animals. At this time point most of the TEBs have differentiated into lobuloalveolar cells. Sampling at various times prior to 50 days of age would answer the question of whether exercise altered the rate of development ie. accelerated or retarded the morphological development of the mammary gland. Although we did not control for sampling at different times of the estrous cycle, it is unlikely that this would affect mammary gland differentiation. In studies using exercise intensities similar to ours, both Thompson et al. (1989) and Yedinak (1988) saw no effect of exercise on vaginal opening or estrous cycle periodicity. Because prolactin is involved in ductal maintenance and mammary gland development, we assessed the effects of exercise on basal serum prolactin levels. In parallel with the lack of observed changes in development, there was also no difference in serum prolactin levels between sedentary and exercised rats. The serum was collected 24 hours after the last bout of exercise in order to get a true representation of basal level. A more accurate assessment of prolactin requires knowledge of estrous cycle status as circulating prolactin levels increase 8 to 10 fold during proestrus compared to diestrus. Prolactin is but one hormone that is involved in the development of the rat mammary gland. Other hormones such as estrogen, progesterone and growth hormone need to be assessed.

One of the reasons we chose to look at the effects of exercise on mammary gland development was that energy balance studies which altered dietary intake have shown effects on sexual development in rats. The linking hypothesis is that if energy restriction affects development then the converse, increased energy expenditure may show similar effects. Arts et al. (1992) studied the effects of an energy restricted low fiber diet on mammary gland development and proliferation. They found a delayed vaginal opening, delayed mammary gland development and decreased proliferation in terminal end bud (TEB) cells but not ductal cells. Lok et al. (1990) used mice to study the effects of caloric restriction on mammary epithelial cell proliferation. As in the rat studies, dietary restriction resulted in decreased proliferation. Park et al. (1994) found energy restriction delayed vaginal opening. In each of these studies there was a significant decrease in body weight. In our study there was no difference in body weight between rats which were exercised and those that remained sedentary and this may have contributed to the lack of an effect of exercise on mammary gland development.

Proliferation rates of mammary epithelial cells from exercised and sedentary rats were determined following four weeks of moderate intensity exercise training. We used the four day continuous BrdU labeling method to control for the estrous cycle and to increase sensitivity of the assay. In comparing BrdU labeling and ^{3}H thymidine labeling, Eldridge et al. (1990) found similar results with the two methods. Eldridge et al. (1990) also compared single injection vs. continuous administration and found the continuous methods to be more sensitive. Exercise increased the proliferation rates of lobuloalveolar cells in all intensity/durations tested. The importance of this increase is unkown as it is hypothesized that TEBs and not lobuloalveolar cells are the targets of carcinogenic attack (Russo et al. 1982).

The susceptibility of a cell to undergo malignant transformation depends on factors such as rates of DNA synthesis, cell proliferation, the length of G1 and S phases of the cell cycle and the cellular competency in DNA excision repair (Russo et al. 1982). These factors determine the ability of a carcinogen to bind to DNA and induce damage. In the next chapter, we address the question of whether or not there is increased damage in the mammary gland DNA derived from exercised rats.

Chapter IV Nitrosomethylurea Distribution, binding and induced DNA damage

4.1 Introduction

Carcinogenesis is a multistage process involving at least three steps: initiation, promotion and progression. Initiation is characterized by irreversible permanent change(s) in the DNA of target cells. Stable alteration of the DNA requires binding of a carcinogen to the DNA with maximal binding occurring during DNA synthesis (Russo and Russo 1991). DNA damage by carcinogens occurs mostly during the S phase of the cell cycle. If the damage is not repaired during the G1 phase, the damaged DNA is passed on to the daughter cells (Berenblum 1976; Frei and Harsano 1967; Kakunaga 1975; Marquardt et al. 1979). In order for a carcinogen to be effective it must reach the target cell and cause DNA damage. Nitrosomethylurea (NMU) is a direct acting carcinogen commonly used in rodent models to induce mammary tumorigenesis. Iv or ip. injections reproducibly and specifically induce mammary tumors. In this model, NMU exhibits a dose response with increased tumor incidence and decreased latency with increasing doses (Verdeal et al. 1982; McCormick et al. 1981; Thompson and Adlakha 1991; Anisimov 1988). Despite this finding, few investigators have examined the mechanism(s) of NMU induced tumorigenesis and no *in vivo* studies on mechanisms have been done.

A number of questions arise as to the process of NMU action. Because a dose response in tumor induction has been seen with increasing doses of NMU, we wanted to determine if there was both increased delivery of NMU to the mammary gland and binding to the DNA with increasing dosage of carcinogen. Secondly, we wanted to determine if there was increased DNA damage as indicated by the nick translation assay with an increasing dosage of NMU. The rat has six pairs of mammary glands with different regional locations:

cervical, thoracic, abdominal and inguinal (see Figure 1.4). Investigators have found more tumors induced in the cervical-thoracic chain than in the abdominal-inguinal chain (Thompson and Adlakha 1991; Russo and Russo 1987; Russo and Russo 1988). A question which arises is does gland location affect NMU binding and/or DNA damage?

In the previous chapter, the proliferation rate of mammary gland lobuloalveolar epithelial cells from exercised rats was significantly increased compared to those from sedentary rats. Thus the time available for DNA repair would have decreased and the number of cells susceptible to carcinogen attack would have increased. However, it is not known if this increased proliferation rate observed in exercised rats would lead to an increase in NMU induced DNA damage in mammary epithelial cells. NMU is a direct acting alkylating agent which spontaneously decomposes first to a diazonium intermediate and then to nitrogen and a carbonium ion (Margison and O'Connor 1978). Although exercise would not affect the metabolism of NMU it may alter its distribution through the bloodstream and to the target organ, the mammary gland. Secondly we have shown exercise alters proliferation rate of lobuloalveolar cells, this may affect methylation of mammary gland DNA by NMU.

Therefore, the purposes of this study were to determine 1) the binding characteristics of NMU at three different doses in each of the regional glands, cervical-thoracic, abdominal and inguinal, 2) DNA damage induced by NMU at three different doses in each of the regional glands, and 3) the effects of exercise on DNA damage in the individual mammary glands.

4.2 Methods

In experiment one, the binding and distribution of ^{3}H NMU was assessed in sedentary rats. In experiment two, nick translation activity was assessed in mammary glands from sedentary and exercised rats.

4.2.1 Animals

Experiment one: Female Sprague Dawley rats were obtained from Charles River at 45 days of age. At fifty days of age the rats were randomly assigned to one of three groups (n=5) corresponding to either a low dose (25 mg NMU/kg) moderate dose (37.5 mg NMU/kg) or high dose (50 mg NMU/kg) of ^{3}H NMU.

Experiment two: Twenty one day old female Sprague-Dawley rats were obtained from Charles River Labs. Upon arrival, the rats were randomly divided into two groups, sedentary (n=24) and exercised (n=12). Both groups were maintained in the same room on a twelve hour light dark cycle and fed laboratory rodent diet 5001 ad libitum (Ralston Purina). The rats were exercised for four weeks as per the protocol for MS exercise group in chapter 3 (final workload 18 m/min.; 60 min/day).

4.2.2 NMU preparation and administration

NMU was administered as described in Thompson and Adlakha (1991). Briefly, NMU was purchased from Ash Stevens and dissolved in 0.9% NaCl solution pH 4 (acidified with acetic acid) to a concentration of 14mg/ml. Each vial of NMU was used within 25 minutes of preparation. For experiment one, each animal received an ip. injection of N-[^{3}H] methyl-N-nitrosourea (^{3}H NMU) at a dose of either 25, 37.5 or 50 mg NMU/kg at fifty days of age. For every injection a tube of ^{3}H NMU was prepared from x mls cold plus 200 µl ^{3}H NMU (1 mCi/ml). X mls was determined by how much of the 14mg/ml stock NMU

was required in order to administer the appropriate dose of either 25, 37.5 or 50 mg NMU/kg. Each animal was weighed and the appropriate amount of NMU was delivered with an ip. injection. In experiment two, twenty four hours after the last bout of exercise (at fifty days of age), the exercised rats were given injections of either 37.5 or 50 mg NMU/kg body weight ip. (n=6/dose). The sedentary rats were given injections of either 25, 37.5 or 50 mg NMU/kg body weight ip. (n=8/dose). The injections took place at a rate of one animal every seven minutes.

4.2.3 Tissue collection

Two hours post injection the rats were euthanized by CO_2 asphyxiation. The rats were taken in the order they were injected, one every seven minutes. Mammary glands two through six were removed from the left and right sides with glands two and three pooled (thoracic), and glands four and five pooled (abdominal). The glands were frozen in liquid nitrogen and kept at -80^{0}C until analysis. Mammary glands from the left and right sides were analyzed separately and the results were averaged. In experiment two, rats were taken as pairs (n=1) and glands were removed and pooled as above. For experiment one portions of the liver were removed and frozen in liquid nitrogen and kept at -80^{0}C until analysis. Whole blood was collected from cardiac puncture and centrifuged at 600 x g for fifteen minutes. The serum was removed and stored at -20^{0}C until analysis.

4.2.4 DNA isolation

Frozen mammary glands were pulverized under liquid nitrogen. The glands were placed in digestion buffer (100mM NaCl; 10 mM Tris pH 8.0; 0.5% SDS; 25 mM EDTA pH 8.0) at a concentration of 1.25 mls/100mg. The glands were incubated for fifteen minutes at 50^{0}C in a shaking waterbath. 25 µl RNase A (11mg/ml) was added to the digestion mixture and

incubated for 100 minutes in a 50^{0}C shaking waterbath. Then 500 µl proteinase K (1.6 mg/ml) was added and the mixture was incubated overnight at 50^{0}C in a shaking waterbath. The digested glands were removed from the waterbath and cooled on the bench for five minutes. 100 µl aliquots of homogenate were taken for determination of ^{3}H NMU and its metabolites in whole mammary gland and set aside. 1.5 ml of 7.5M ammonium acetate was added to each sample which were then spun at 7700 x g for 40 minutes. The supernatant was decanted immediately and extracted twice with Sevag (Chloroform:isoamyl alcohol 24:1) spinning at 7700 x g for 5 minutes each time. The DNA was precipitated from the aqueous phase with cold 100% ethanol. The clump of DNA was transferred to an eppendorf tube and washed twice with 70 % ethanol. The pellet was air dried and resuspended in 750 µl TE buffer. In order to dissolve the DNA, the samples were incubated in the 50^{0}C shaking waterbath overnight. DNA concentration was determined spectrophotometrically as follows: A 10 µl aliquot of each sample was added to 990 µl TE buffer and read at 235, 260 and 280 nm. Each sample was done in duplicate. To determine the amount of ^{3}H methylation of each sample 10 ml Hionic Fluor was added to each sample and counted in the Beckman scintillation counter. For calculations see appendix.

4.2.5 Liver

The frozen liver samples were homogenized in 50 mM Tris Cl pH 8.0. The samples were added to 10 mls Ecolite and counted in the Beckman scintillation. The counts represent the presence of ^{3}H NMU and its metabolites. For calculations see appendix.

4.2.6 Serum

200 µls of each sample was added to 10 mls Ecolite and counted in the Beckman scintillation counter. . The counts represent the presence of ^{3}H NMU and its metabolites. For calculations see appendix.

4.2.7 Epithelial cell isolation

The mammary glands were prepared for disaggregation by mincing with sterile scapel blades in petri dishes. The minced glands were incubated overnight at $37^{0}C$ in disaggregation buffer (95% DME medium; 10mM HEPES buffer; 5% FCS; insulin 5 µg/ml; 0.15% collagenase). The epithelial cells were collected by centrifugation and washed twice with DME. The cells were counted in a haemocytometer.

4.2.8 Nick translation assay

This assay is modified from Nose and Okamoto (1983). The epithelial cell pellet was resuspended in 1ml of solution 1 (0.25 M sucrose; 0.1 M Tris HCl pH 7.4; 10 mM $MgCl_2$; 0.5 mM DTT). 100 µg/ml lysolecithin was added just before use. The samples were incubated on ice for five minutes and centrifuged at 1500 x g for five minutes. The pellets were resuspended in 200 µl nick translation buffer (50 mM Tris HCl pH 7.4; 5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 50 µg/ml BSA; 0.05 mM each dATP, dGTP, dTTP and 5 µCi/ml ^{3}H dCTP; *E. coli* DNA polymerase I was added to 40 units/ml). The samples were incubated at room temperature for thirty minutes. Using the Millipore manifold, 1MM discs were prewetted with 2% pyrophosphate. Samples were applied to the discs washed with 5% TCA, 1% pyrophosphate and rinsed with ethanol. The discs were dried and

transferred to scintillation vials containing 10 mls scintillation cocktail . The samples were counted in the Beckman scintillation counter. For calculations, see appendix.

4.2.9 Statistical analysis

Results were analyzed by one way analysis of variance. Posthoc analysis was performed using Tukey's test.

4.3 Results

The presence of ^{3}H NMU and its metabolites two hours post injection was determined in serum, liver and mammary gland tissue. The amount of ^{3}H NMU and its metabolites in the serum and liver was greater with increasing amounts of injected ^{3}H NMU (Figures 4.1 and 4.2).

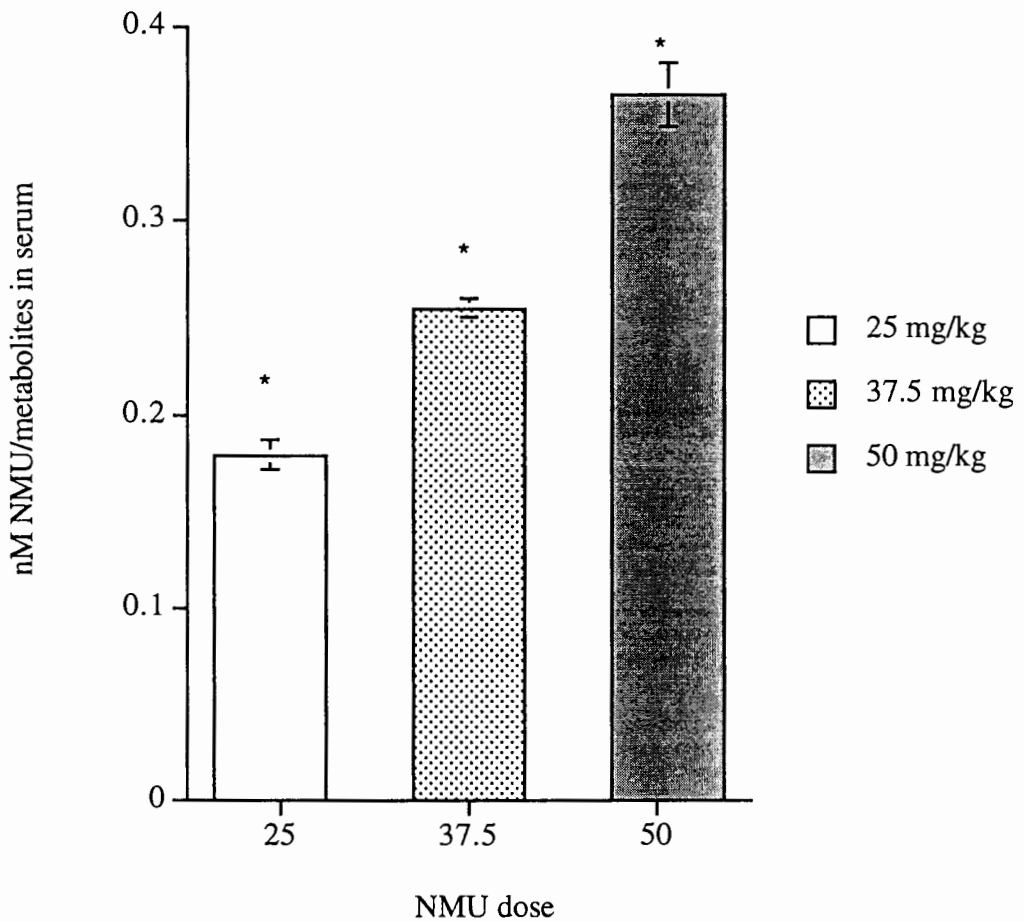


Figure 4.1. Concentration of ^{3}H NMU/metabolites (nM) in serum of rats injected with 25, 37.5 or 50 mg/kg ^{3}H NMU. * There is a significant difference between all three doses ($p<.05$). Values are expressed as means $\pm$ S.E.

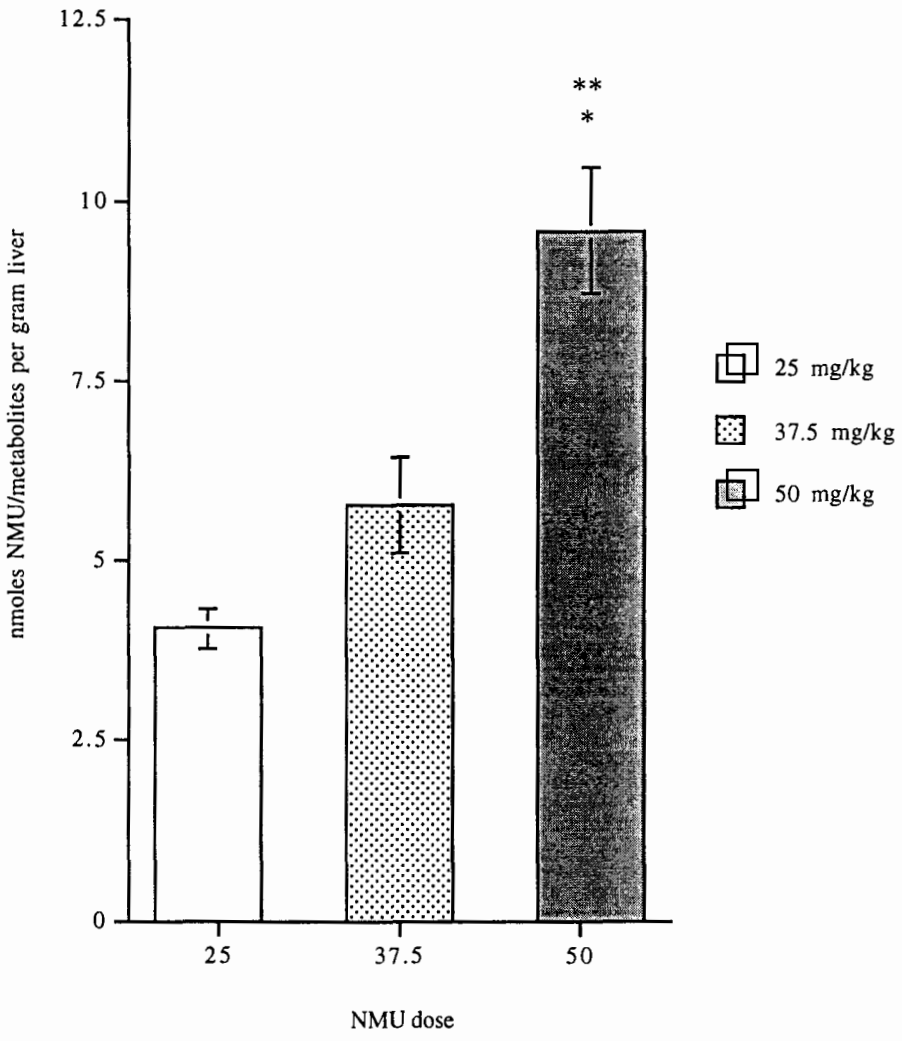


Figure 4.2. Concentration of ^{3}H NMU/metabolites (nmoles NMU per gram liver) in liver samples from rats injected with 25, 37.5 or 50 mg/kg ^{3}H NMU. There is a significant difference between all three doses ($p<.05$) except for 25 mg NMU/kg vs. 37.5 mg NMU/kg. Values are expressed as means $\pm$ S.E. * Significantly different from 25 mg NMU/kg. ** Significantly different from 37.5 mg NMU/kg.

Figure 4.3A demonstrates the amount of NMU/metabolites per gram of mammary tissue with increasing doses of NMU as a function of gland location: thoracic glands (2,3), abdominal glands (4,5) and inguinal glands (6). In general, the amount of NMU/metabolites detected in the tissue homogenate was not proportional to the amount of NMU injected into the animal. In the thoracic and abdominal glands, there was no difference between any of the doses tested. In the inguinal mammary gland, there was a significant increase in the amount of ^{3}H NMU/metabolites bound when 50 mg NMU/kg is administered compared to 37.5 mg NMU/kg. In analyzing the amount of ^{3}H methylation per mg DNA, there was a positive trend with increasing dose in all glands with 50 mg NMU/kg significantly higher than 25 mg NMU/kg(Figure 4.3B). The amount of methylation per mg DNA was increased approximately four times with a dose of 50 mg NMU/kg compared to 25 mg NMU/kg in the cervical and inguinal glands. In the abdominal and inguinal mammary glands there was also a significant difference between 50 mg NMU/kg and 37.5 mg NMU/kg. For the abdominal glands, the increased methylation appears to be linear. To determine if the glands took up different amounts of circulating ^{3}H NMU/metabolites, the ratio of ^{3}H methylation per mg DNA: ^{3}H NMU/metabolites in serum was calculated. With the exception of the inguinal glands, there was no difference in the methylation per mg DNA when normalized for serum concentration. In the inguinal mammary glands, the ratio for 50 mg NMU/kg was significantly higher than both 25 mg NMU/kg and 37.5 mg NMU/kg (Figure 4.4A).

The nick translation assay was used as an assessment of DNA damage. The pattern within glands was similar to that observed for serum normalized NMU binding to mammary gland DNA. There was no difference between doses except for the inguinal glands where there was a significant difference between 37.5 and 50 mg NMU/kg (Figure 4.4B).

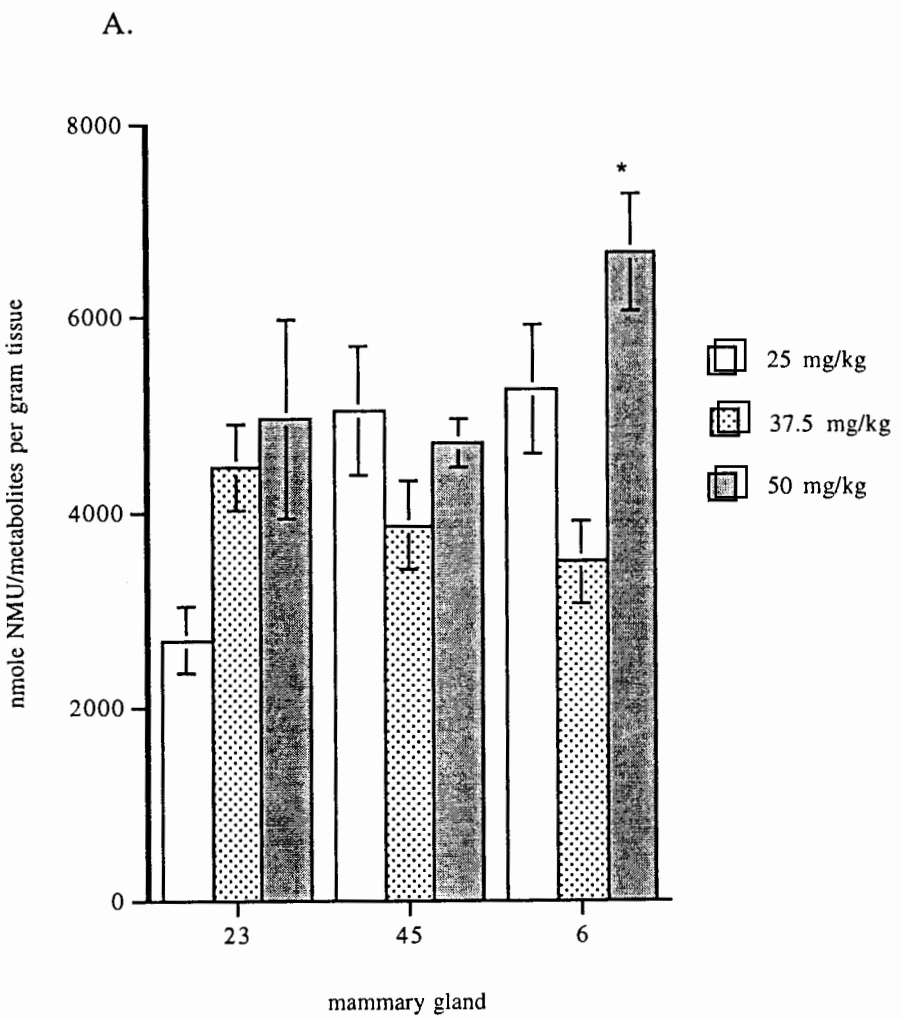


Figure 4.3A. Concentration of ^{3}H NMU/metabolites (nmole NMU per gram tissue) in mammary gland homogenates comparing between the three doses 25, 37.5 or 50 mg/kg ^{3}H NMU within each mammary gland pair 2,3; 4,5; 6. * There is a significant difference between 50 mg NMU/kg and 37.5 mg NMU/kg ($p<.05$).

B.

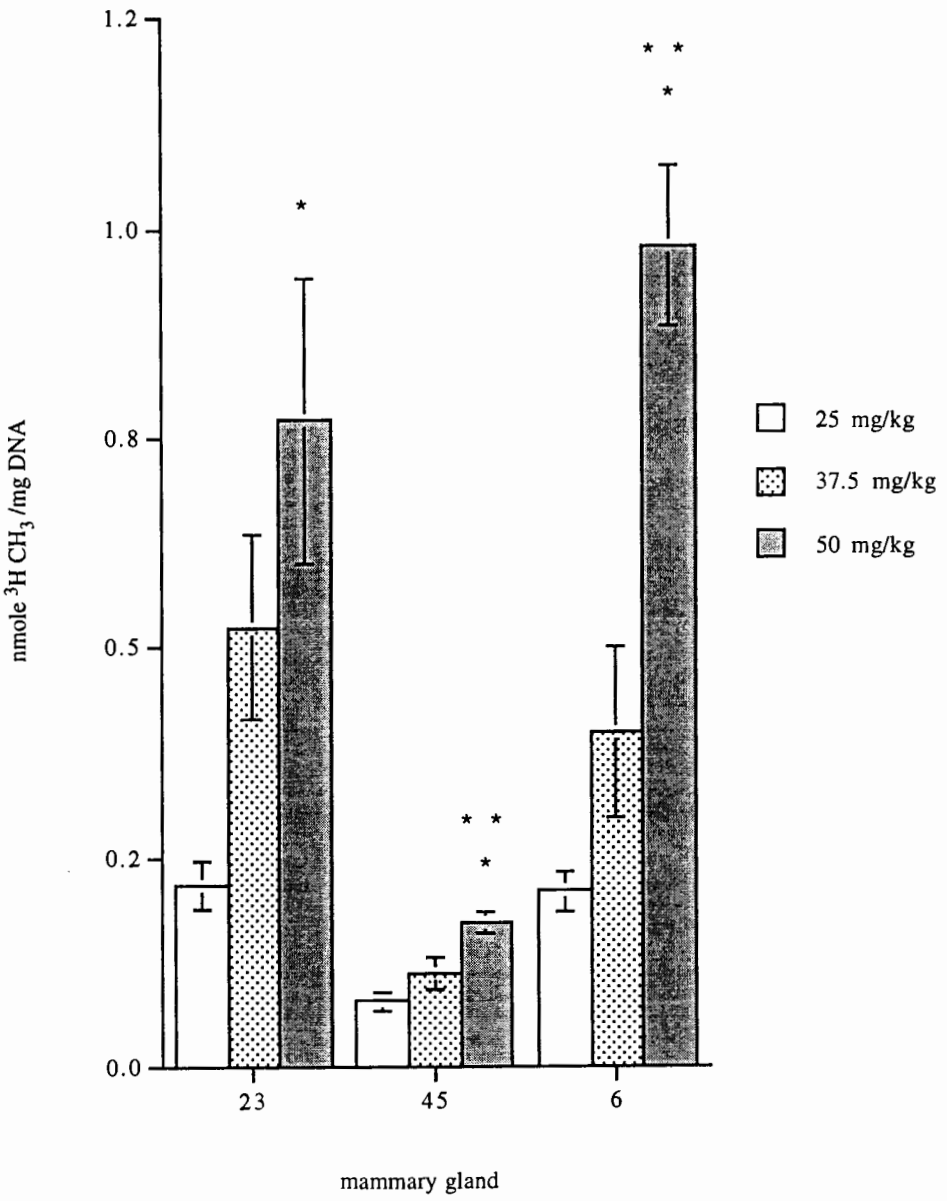


Figure 4.3B. Concentration of methylation (nmoles ^{3} H CH_3 /mg DNA) in mammary gland DNA comparing between the three doses 25, 37.5 or 50 mg/kg ^{3}H NMU within each mammary gland pair 2,3; 4,5; 6. *There is a significant difference between 25 mg NMU/kg and 50 mg NMU/kg; **There is a significant difference between 37.5 mg NMU/kg and 50 mg NMU/kg ($p<.05$). Values are expressed as means $\pm$ S.E.

A.

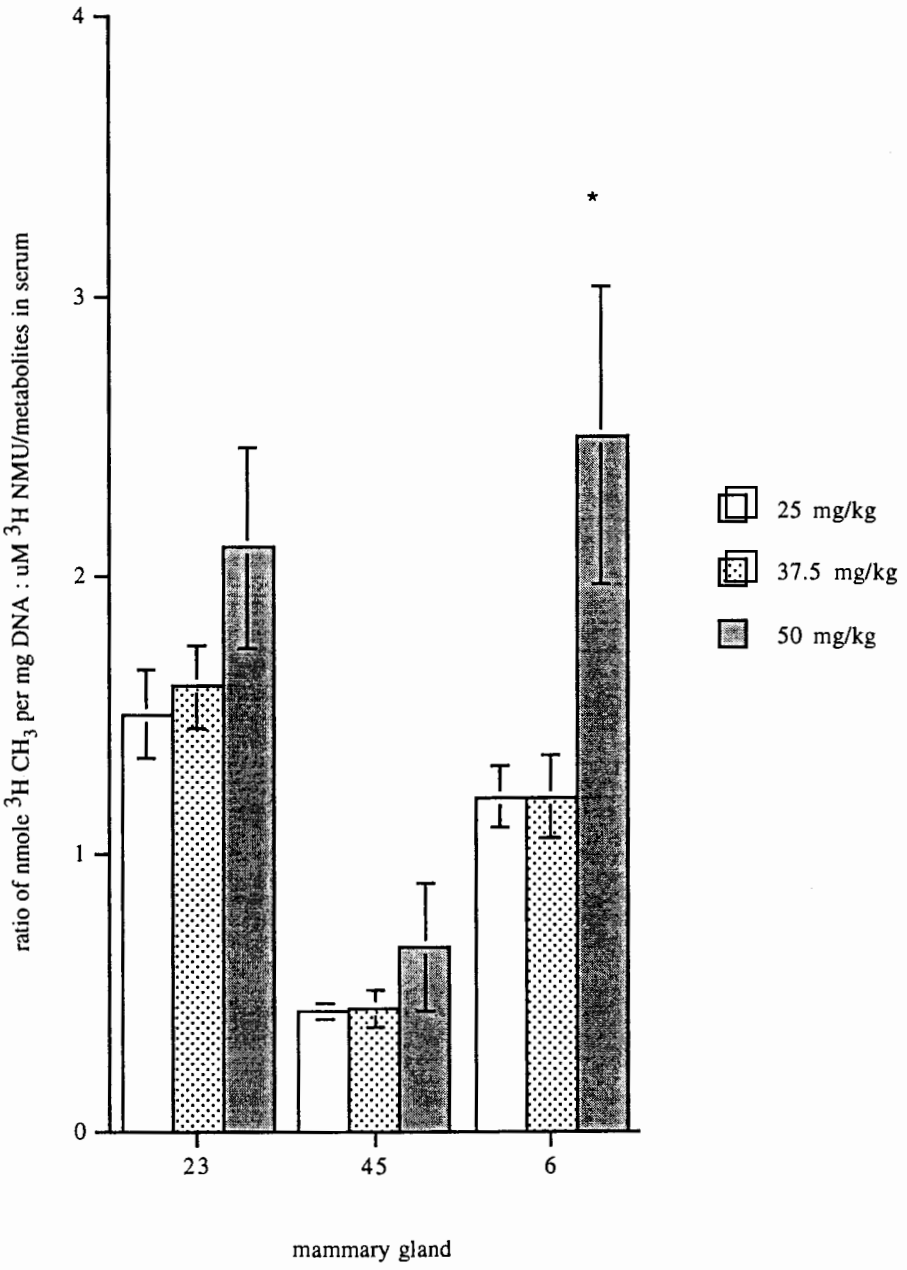


Figure 4.4A. Ratio of methylation/mg DNA: mM ^{3}H NMU/metabolites in serum comparing between the three doses 25 37.5 and 50 mg NMU/kg within each mammary gland pair. *There is a significant difference between 50 mg NMU/kg and the other two doses ($p>.05$).

B.

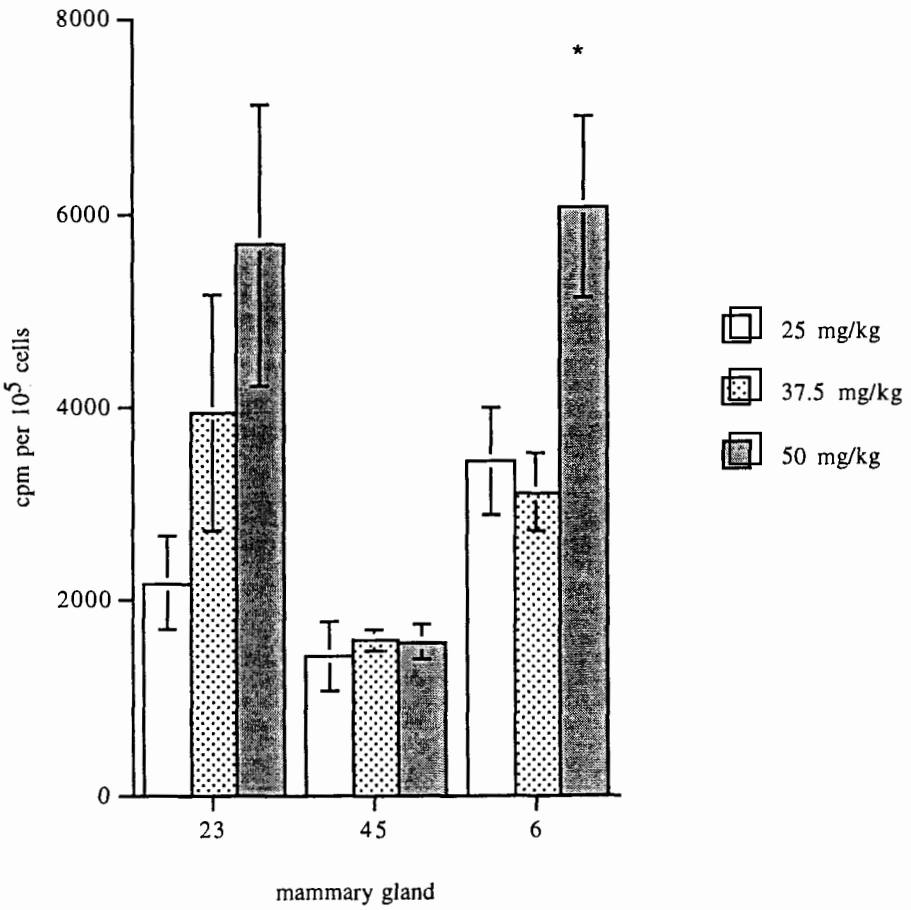


Figure 4.4B. The comparison of cpm per 10^5 cells between the three doses 25, 37.5 50 mg NMU/kg within each of the three gland pairs 2,3; 4,5; 6 in sedentary rats. * There is a significant difference in the amount of nick translation between a dose of 37.5 mg NMU/kg and 50 mg NMU/kg ($p<.05$). Values are expressed as means $\pm$ S.E.

The results for mammary gland tissue were also analyzed within doses to determine the relationship between glands. In assessing the difference between glands, there was no difference in the amount of NMU/metabolites taken up by the individual mammary glands with two minor exceptions (Figure 4.5A). At a dose of 25 mg NMU/kg, there was significantly less ^{3}H NMU/metabolites in the homogenate of thoracic mammary glands compared to abdominal and inguinal mammary glands. For a dose of 50 mg NMU/kg, inguinal mammary glands contained significantly more ^{3}H NMU/metabolites in the homogenate compared to abdominal mammary glands. When analyzing the amount of ^{3}H CH_3 per mg DNA, a clear pattern emerges. Abdominal mammary glands bound significantly less ^{3}H CH_3 per mg DNA at all doses tested (Figure 4.5B). As well, the ratio of ^{3}H CH_3/mg DNA: ^{3}H NMU/serum was significantly less in abdominal mammary glands for all three doses (Figure 4.6A). Figure 4.6B demonstrates that nick translation activity was significantly decreased in the abdominal mammary glands.

The nick translation activity at doses of 37.5 and 50 mg NMU/kg was examined in mammary epithelial cells from sedentary and exercised rats. There was no difference in activity between the two groups (Figures 4.7A and 4.7B).

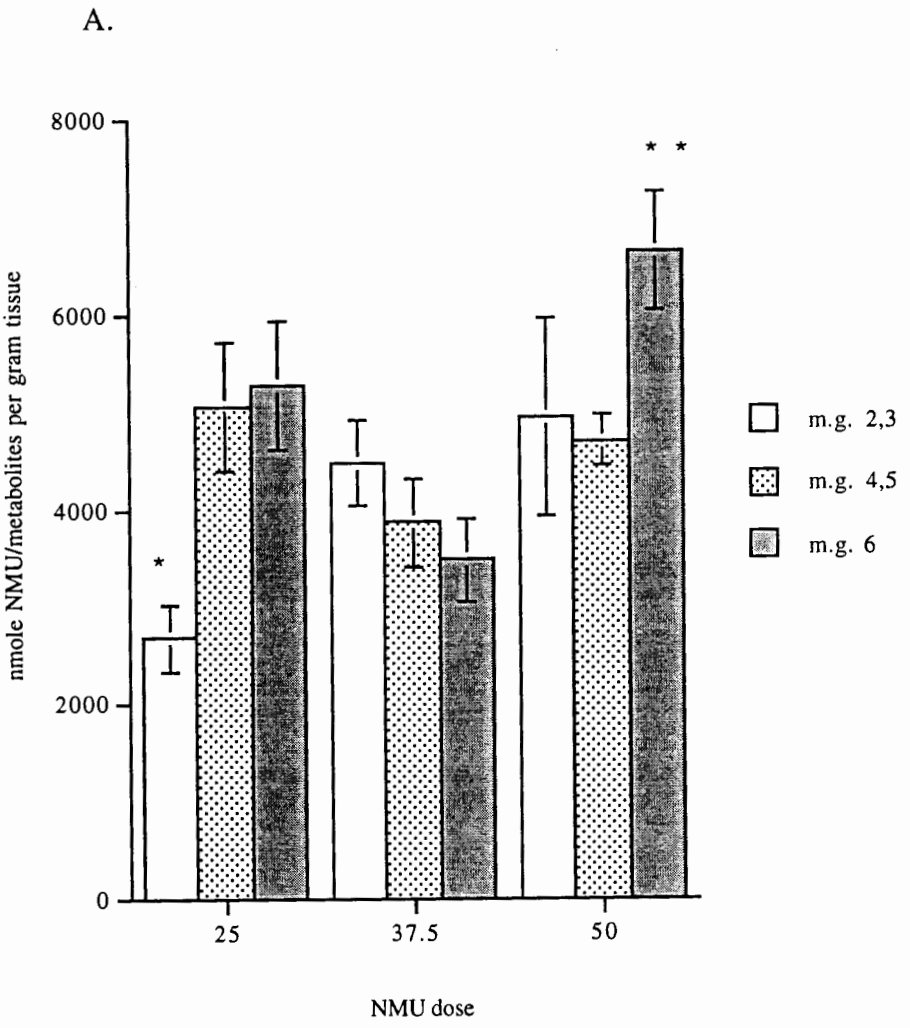


Figure 4.5A. Concentration of ^{3}H NMU/metabolites (nmole NMU per gram tissue) in mammary gland homogenates comparing between the three glands 2,3; 4,5;6 within each dose 25, 37.5 or 50 mg/kg ^{3}H NMU. * The amount of ^{3}H NMU in mammary gland 2,3 homogenate is significantly different from mammary glands 4,5 and mammary gland 6 with a dose of 25 mg NMU/kg; **the amount of ^{3}H NMU in mammary gland 6 is significantly different from mammary gland 4,5 ($p<.05$).

B.

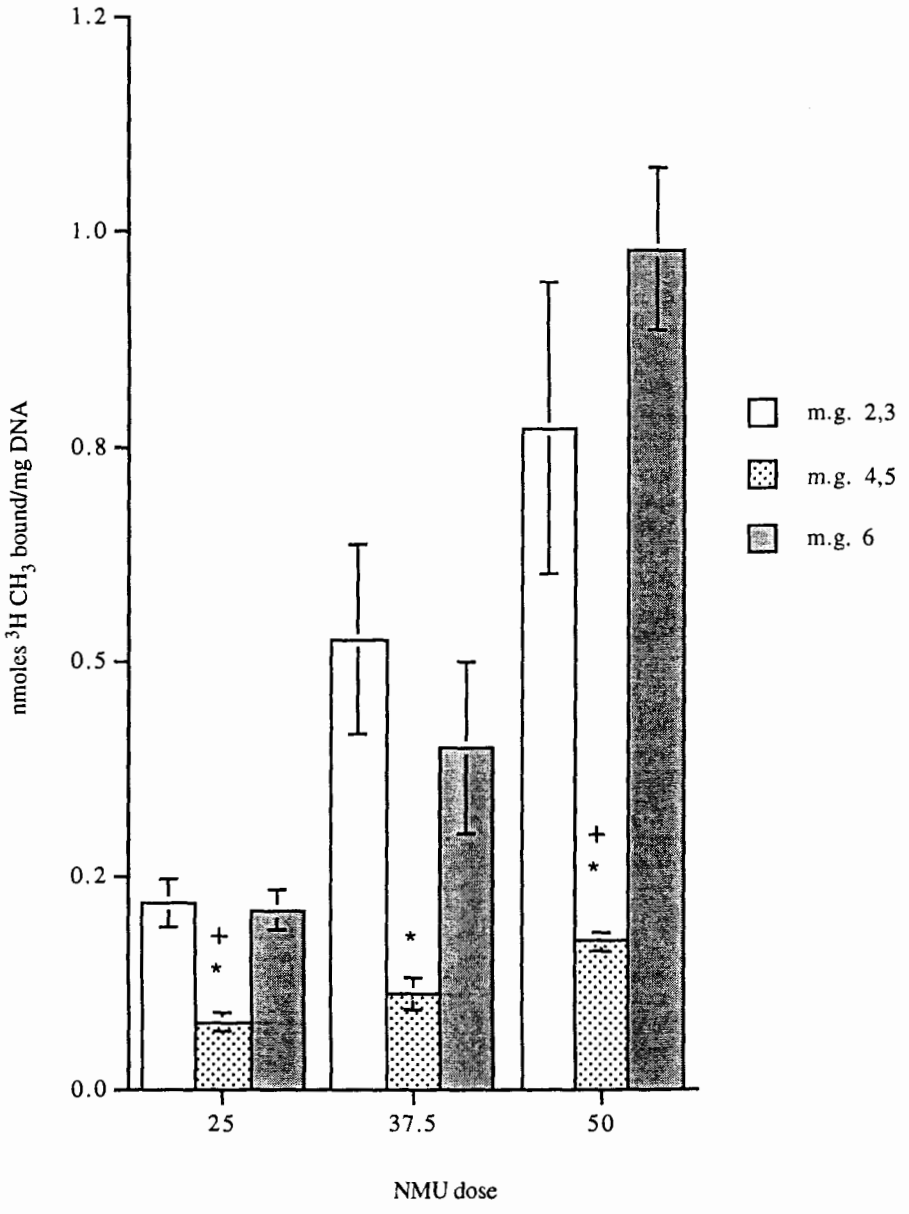


Figure 4.5B. Concentration of methylation (nmoles ^{3} H CH$_3$ /mg DNA) in mammary gland DNA comparing between the three glands 2,3; 4,5; 6 within the three doses 25, 37.5 or 50 mg/kg ^{3}H NMU. * Mammary gland 4,5 is significantly different than mammary gland 2,3; $^+$Mammary gland 4,5 is significantly different than mammary gland 6 ($p<.05$). Values are expressed as means $\pm$ S.E.

A.

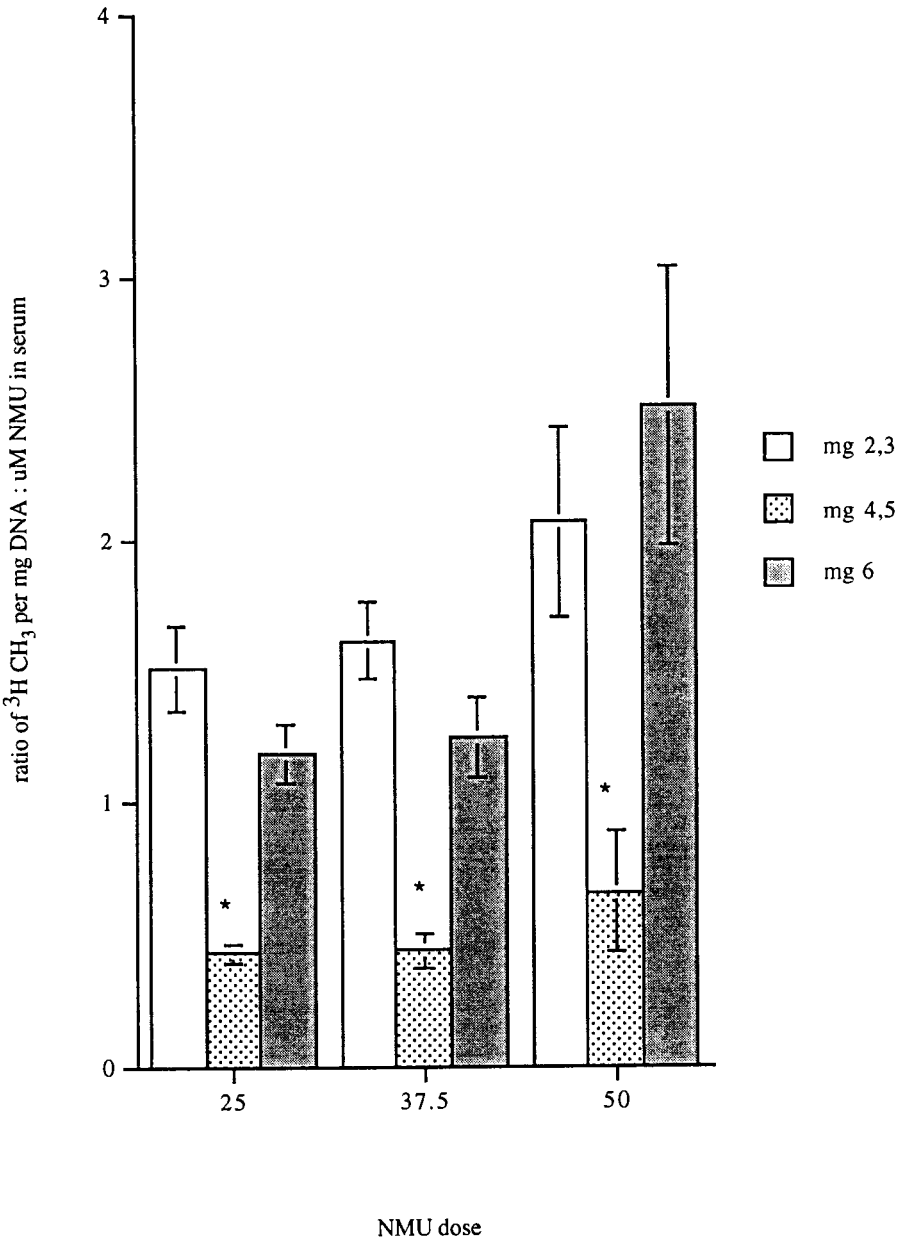


Figure 4.6A. Ratio of nmole ^{3}H CH_3 /mg DNA: mM ^{3}H NMU in serum comparing between the three glands 2,3; 4,5; 6 within each dose 25 37.5 and 50 mg NMU/kg. *Mammary gland 4,5 is significantly different from mammary gland 2,3 and mammary gland 6 ($p<.05$).

B.

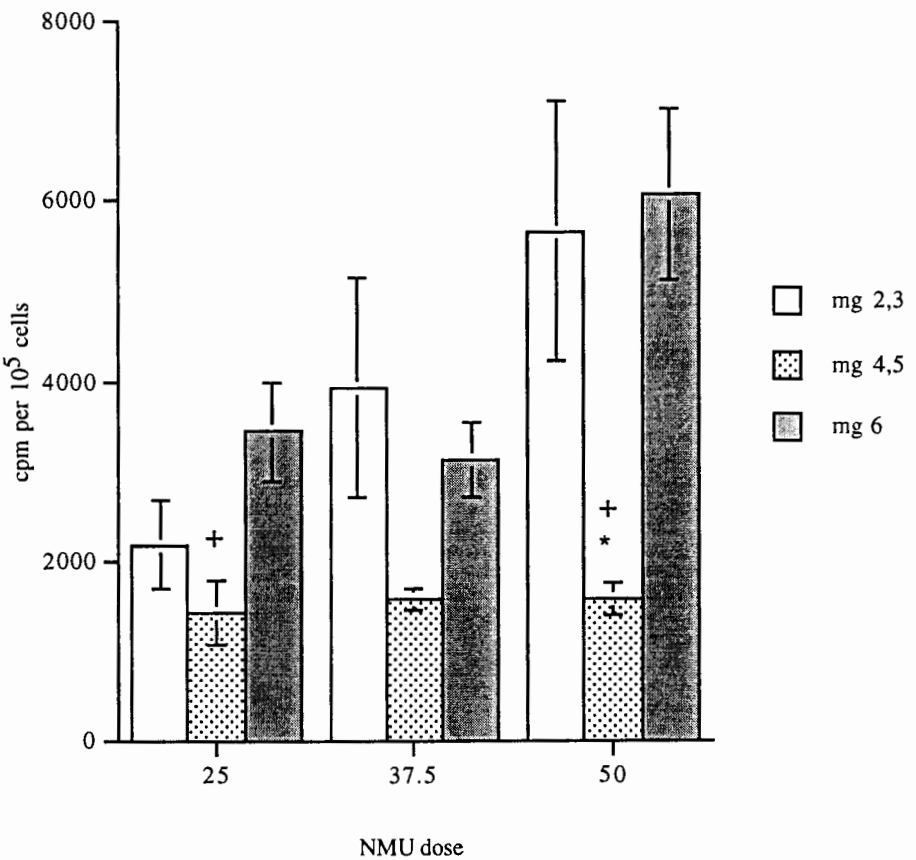


Figure 4.6B. The comparison of cpm per 10^5 cells between the three gland pairs 2,3; 4,5; 6 within each of the three doses 25, 37.5 50 mg NMU/kg in sedentary rats. * There is a significant difference in the amount of nick translation between a mammary glands 2,3 and 4,5 ($p<.05$). $^+$ There is a significant difference between mammary glands 4,5 and 6 ($p<.05$). Values are expressed as means $\pm$ S.E.

A. 37.5 mg NMU/kg

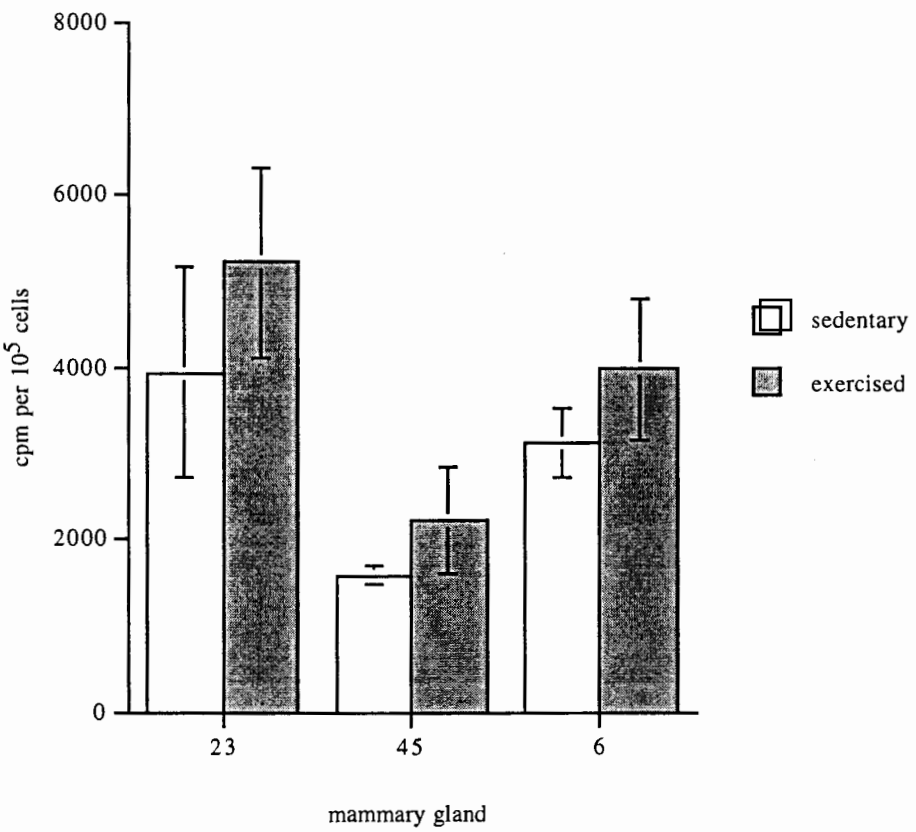


Figure 4.7A. The comparison of cpm per 10^5 cells between sedentary and exercised rats within the three glands 2,3; 4,5; 6 at a dose of 37.5 mg NMU/kg. There are no differences ($p>.05$).

B. 50 mg NMU/kg

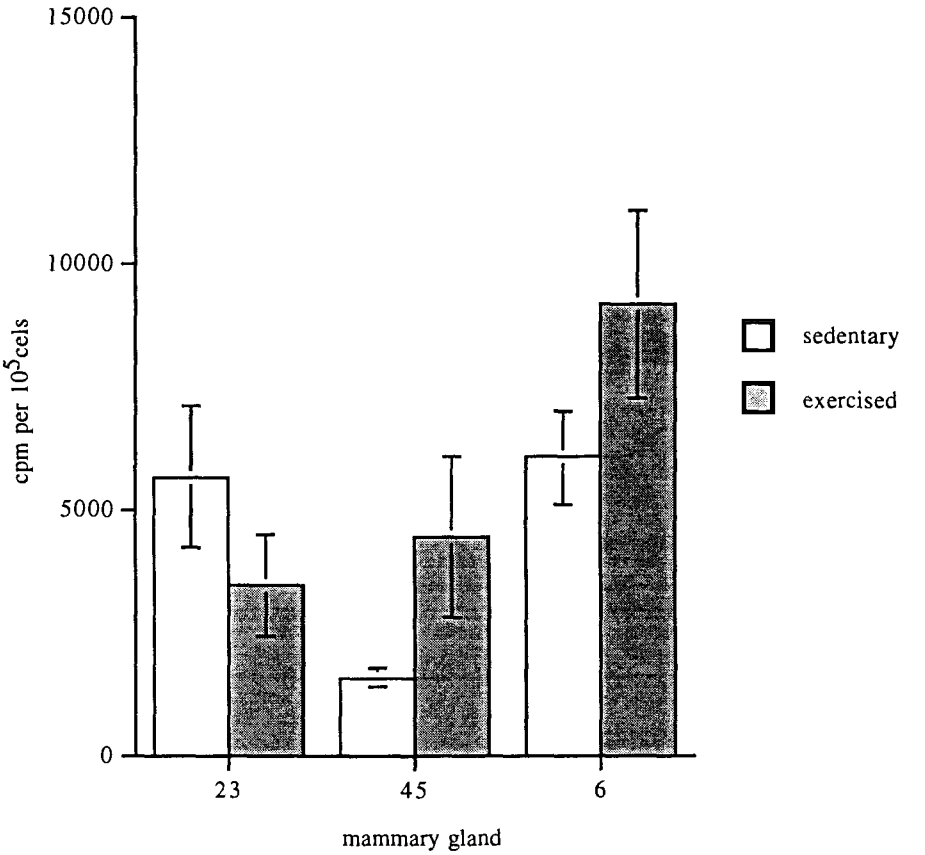


Figure 4.7B. The comparison of cpm per 10^5 cells between sedentary and exercised rats within the three glands 2,3; 4,5; 6 at a dose of 50 mg NMU/kg. There are no differences ($p>.05$). Values are expressed as means $\pm$ S.E.

4.4 Discussion

The results of these experiments yield a number of interesting new observations relating to the NMU induced carcinogen model of mammary tumorigenesis. Increasing the amount of NMU injected increased the NMU/metabolites concentration in both the serum and liver. Methylation of mammary gland DNA was directly related to the NMU levels in the serum although not in a linear fashion. Abdominal mammary glands behaved differently than either thoracic or inguinal mammary glands by incorporating significantly less methylation. Patterns of NMU binding were mimicked in the patterns of DNA damage induced by NMU. Exercise had no effect on mammary gland DNA damage.

NMU is utilized as a mammary carcinogen in mice and rat studies for a number of reasons: 1) it is direct acting, requiring no activation by the host; 2) the hormone dependency of the tumors is similar to the human disease 3) the tumors are of ductal origin and have predominantly carcinomatous histopathology (Welsch 1985). NMU is administered to the animal with either an iv. or ip. injection. Thompson et al. have observed similar tumor induction and incidence with the two methods (Thompson and Adlakha 1991). Therefore we chose to use the ip. injection because of its relative simplicity. NMU dose response induction of mammary tumors has been studied using both iv. and ip. injections and single or multiple injections (Verdeal et al. 1982; McCormick et al. 1981; Thompson and Adlakha 1991). With any method there is an increase in tumor incidence and a decrease in latency with increasing dose. We wanted to test the dose response of NMU distribution in the rat. With an ip. injection the NMU solution is injected into the peritoneal cavity and absorbed by the venous system where it is carried back to the heart. The NMU is then distributed to the rest of the body via the arterial system. Using three doses of NMU (25, 37.5 and 50 mg NMU/kg), we saw a linear increase in the presence of NMU/metabolites in the serum. This suggests that as you increase the injection dose of NMU, the amount of NMU

available to various tissues is increased. The amount of mammary gland DNA methylation increased with increasing dose, although it does not appear to be in a linear fashion for the cervical and inguinal glands. This suggests the NMU incorporation is specific for DNA and is dose dependent. The fact that the increase is not linear (four times as DNA methylation at a dose of 50 mg NMU/kg compared to 25 mg NMU/kg) suggests that at 50 mg NMU/kg the system may be overloaded.

The dose of 50 mg NMU/kg is typically used in NMU induced mammary tumor studies because of rapid induction and multiple tumors. However this is not a true representation of the human disease in which there is typically only one tumor and multiple tumors arise from metastases rather than de novo tumors. A dose as high as 50 mg NMU/kg may be overloading the system and the effects of any intervention may be masked. Eight weeks post carcinogen administration the incidence of palpable tumors in rats administered 50 mg NMU/kg is 100% (Thompson and Adlakha 1991). The number of tumors per rat (multiplicity) at the end of the 28 week experimental period is 5.4 cancers/rat (Thompson and Adlakha 1991). By lowering the dose to 37.5 mg/kg, the incidence of of palpable tumors was 60% at 8 weeks and reached 96% at 20 weeks post carcinogen administration (Thompson and Adlakha 1991). The multiplicity at this dose is 3.4 cancers/rat (Thompson and Adlakha 1991). Due to the adequate tumor induction with 37.5 mg NMU/kg and its consistent behavior in terms of NMU binding we suggest it is a more feasible dose to use in mammary tumor studies. In order to generate a true dose response curve, more doses between 0 and 50 mg NMU/kg would need to be tested with larger sample sizes to decrease the within group variability.

With a minor exception, there was no difference in DNA methylation when normalized for NMU serum concentration. In other words the amount of NMU taken up by the mammary gland and DNA methylation was proportional to the amount of NMU circulating in the

blood. One would expect DNA damage to increase in a similar fashion to the increased DNA methylation. However, the nick translation results were not clear cut. One possible explanation is that the endogenous endonuclease repair mechanism may be a limiting factor in producing nicks in the damaged DNA. In the nick translation assay, the exogenous DNA polymerase recognizes nicked DNA. Another possibility is the variability and different behavior of mammary glands. Each gland location gave a different pattern between doses. Thoracic and inguinal mammary glands showed increased nick translation with 50 mg NMU/kg, however, abdominal mammary glands showed no difference between any of the doses tested. The amount of mammary gland DNA methylation was significantly less in abdominal glands than the other glands regardless of NMU dose administered. The same pattern was detected in the ratio of nmole $^{3}H\ CH_3$ per mg DNA: μM NMU in serum. The decreased amount of NMU and DNA damage could equate with fewer initiated cells and hence fewer tumors formed. This could explain the observation that more tumors develop in the cervical-thoracic region than in the abdominal-inguinal mammary gland chains irrespective of carcinogen dose (Thompson and Adlakha 1991; Russo and Russo 1987; Russo and Russo 1988). Russo and Russo hypothesized that this is due to the asynchronous development of the glands in different topographic areas: thoracic glands lag behind in development and retain higher concentrations of TEBs (Russo and Russo 1987; Russo and Russo 1988). The TEBs are the structures most susceptible to carcinogenic attack because of their high proliferation rate (Russo and Russo 1987). The cells of TEBs cycle approximately every ten hours, whereas the cells of alveolar buds (ABs) cycle approximately once every twenty eight hours (Russo and Russo 1980). The anatomical location of the mammary glands appears to be important in terms of NMU accumulation and DNA methylation. This suggests that either blood flow is a crucial factor in the delivery of NMU to target organs and/or the morphological and biochemical makeup of the glands is different and affects NMU uptake and subsequent methylation.

The lower nick translation activity could also reflect an increase in endogenous repair in abdominal mammary glands. In order to fully assess DNA repair in the mammary glands, a time course study would need to be conducted.

The main methylated product formed by NMU is O^6 methylguanine. This product has been detected in codon 12 of the *Ha-ras* gene in mammary gland DNA. This causes a G to A transition and activates the *Ha-ras* gene. Many researchers believe this is an important event in initiation of NMU induced mammary tumorigenesis (Sukamar et al. 1983; Zarbl et al. 1985; Kumar et al. 1990). Zhang et al. (1990) have shown that with increasing dose of NMU there is a decrease in the activation of *Ha-ras.* Our studies have shown that with increasing administration there is increased delivery of NMU to the mammary gland DNA and increased DNA damage occurring. These results taken with those of Zhang et al. suggest mutations other than *Ha-ras* are occurring. NMU dose response studies on the presence of O^6 methylguanine in mammary glands as well as the activity of alkyltransferase (enzyme responsible for removing methyl group) need to be assessed.

Finally we tested the effects of exercise on nick translation. Nick translation activity was not significantly higher in the mammary gland DNA from exercised rats. This does not correspond to the increased proliferation rate that we observed in mammary gland lobuloalveolar epithelial cells from exercised rats (chapter three). The fact that increased proliferation rate in lobuloalveolar cells does not lead to increased DNA damage supports the hypothesis that TEBS and not Abs are the target cells for carcinogenic attack. In order to understand why the increased proliferation rate in the lobuloalveolar cells did not lead to increased DNA damage, DNA repair capacity in the exercised glands needs to be assessed.

Because the mammary glands in different regional locations differed in terms of NMU binding and DNA damage, it is important to determine if it is the behavior of the cells within the gland or a characteristic of the gland itself. One way to assess this would be to conduct these studies on isolated epithelial cells.

Although the amount of DNA methylation increased with increasing dose, there was no difference in nick translation activity (DNA damage) between the doses. The abdominal glands showed consistently lower nick translation activity when compared to the thoracic and inguinal glands. Exercise had no effect on nick translation. In the next chapter we examine the effects of exercise on NMU induced mammary tumor outcome at 37.5 and 50 mg NMU/kg.

4.5 Appendix

4.5.1 Calculating nmoles NMU/mg DNA

In order to calculate nmoles NMU/mg DNA the specific activity of ^{3}H NMU injected into animal must be calculated.

200 µCi ^{3}H NMU plus 0.320 ml cold NMU (14 mg/ml) =4.48 mg ^{3}H NMU.

mmole= 4.48/103= 0.043

200 µCi/0.043mmole= **4.7 mCi/mmole**

Second, the counts of individual mammary gland are converted to dpm's:

cpm = 273.2 H# = 75 CE = 35

dpm = cpm/CE*100 = 780

cpm = counts per minute; H# = difference between quenched sample and unquenched sample; CE = counting efficiency; dpm = disintergrations per minute.

calculation of nmole NMU/mg DNA

DNA sample = 730 ul of 1.48 µg/ul concentration

= 1080.4 µg

$2.2x10^{6}$ dpm = 1 µCi therefore 780 dpm= $3.5x10^{-4}$ µCi

4.7 mCi=1 mmole therefore $3.5x10^{-7}$ mCi=$7.4x10^{-8}$ mmole

$7.4x10^{-8}$ mmole= $7.4x10^{-2}$ nmole

$7.4x10^{-2}$ nmole NMU/1080.4 µg DNA= .07 nmole NMU/ mg DNA

4.5.2 Calculating nmoles NMU/g liver

An example of how nmoles NMU/g liver was calculated is as follows:

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specific activity of ^{3}H NMU injected into animal = 4.9 mCi/mmole

weight of liver sample = 0.0874g

cpm = 1492.6 H# = 88 CE = 33

dpm = cpm/CE*100= 4428.43

calculation of nmole NMU/g liver

$2.2x10^{6}$ dpm = 1μCi therefore 4428.43 dpm= 0.002 μCi

4.9 mCi= 1 mmole therefore $2x10^{-6}$ mCi= $4x10^{-7}$ mmole

$4x10^{-7}$ mmole= $4x10^{-1}$ nmole

$4x10^{-1}$ nmole NMU/0.0874 g liver= 4.58 nmole NMU/g liver

4.5.3 Calculating nM NMU in serum

Serum: 200 uls of each sample was added to 10 mls Ecolite and counted in the Beckman scintillation counter for ten minutes. Sample calculations are as follows:

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specific activity of ^{3}H NMU injected into animal = 5.1 mCi/mmole

200 μl sample of serum

cpm = 127762.5 H#= 95 CE=30

dpm = cpm/CE*100 = 425875- 2515.2 = 423359.8 (control = 2515.2 dpm)

$2.2x10^{6}$ dpm = 1μCi therefore 423359.8 dpm = 0.192 μCi

5.1 mCi = 1 mmole therefore $1.92x10^{-4}$ mCi = $3.7x10^{-5}$ mmole

$3.7x10^{-5}$ mmole = $3.7x10^{-2}$ μmole

$3.7x10^{-2}$ µmole NMU/200 µl serum = 0.000185 µmole/µl

0.000185 µmole/ µl = 0.000185 µM= 0.185 nM

Chapter V The effects of treadmill running during puberty on subsequent NMU induced mammary tumorigenesis

5.1 INTRODUCTION

The association between exercise and breast cancer has been suggested in a number of epidemiological and laboratory studies. Some retrospective studies on former college athletes indicated exercise at an early age affects the lifetime occurrence of breast cancer incidence in a negative way, whereas others have shown no effect (Frisch et al. 1985; Frisch et al. 1987; Paffenbarger et al. 1987). These initial studies have led to a number of animal (rat and mice) studies examining the effects of exercise training on DMBA or NMU induced mammary tumors. There are conflicting results in terms of tumor incidence, latency and multiplicity in these studies (Cohen et al. 1988; Cohen et al. 1991; Lane et al. 1991; Thompson et al. 1988; Thompson et al. 1989). The differences reported in these investigations, at least in part, are likely due to the different exercise intensities, durations, length of training programs, the timing of the exercise program and the amount and type of carcinogen used (Cohen et al. 1988; Cohen et al. 1991; Lane et al. 1991; Thompson et al. 1988; Thompson et al. 1989). Despite the different effects of exercise on tumor outcome, it is apparent that exercise can be used to alter tumorigenesis in the DMBA or NMU-induced rat model (Cohen et al. 1988; Cohen et al. 1991; Lane et al. 1991; Thompson et al. 1988; Thompson et al. 1989). The studies conducted to date have for the most part focused on implementing exercise programs two weeks after the administration of the carcinogen corresponding to the promotion phase of tumor growth.

The question of exercise having a protective effect needs to be addressed from the point of implementing exercise at an early age before the carcinogen has been administered in order to determine if the exercise through some mechanism can place the mammary gland in a

protective state (ie. it is resistant to carcinogenic attack). Although other investigators have studied the effects of exercise both prior to and during carcinogen administration on subsequent tumor development (Yedinak et al. 1988; Thompson 1994), to our knowledge we are the first to exercise rats for only the four weeks prior to carcinogen administration. The effects of exercise on tumor outcome may be dependent upon the timing (pre-carcinogen, pre/post carcinogen) of the exercise training. In this respect, exercise at different times relative to the stage of tumor development may act through different mechanisms to alter tumorigenesis. Since epidemiological studies have shown that exercise early in life may decrease breast tumor development at a later age, we felt it was necessary to investigate this further in an animal model. We therefore conducted two experiments using two different doses of NMU, 50 mg NMU/kg and 37.5 mg NMU/kg respectively, comparing sedentary and exercised rats. A number of studies utilize a dose of 50 mg NMU/kg, which will induce multiple tumors if administered at 50 days of age. One of the questions we addressed was, does the higher amount of NMU override any beneficial effect of exercise?

The purpose of this investigation was to determine the effects of moderate exercise training during peripuberty on subsequent NMU induced mammary tumorigenesis at two doses of NMU.

5.2 METHODS

The effects of exercise prior to carcinogen administration on subsequent tumor development was performed in two separate studies, one with a dose of 50 mg NMU/kg body weight and one with a dose of 37.5 mg NMU/kg body weight.

5.2.1 Animals

For both the 37.5 mg NMU /kg (n=80) and the 50 mg NMU/kg study (n=60), twenty one day old female Sprague-Dawley rats were obtained from Charles River Labs. Upon arrival, the rats were randomly and equally divided into two groups, sedentary and exercised. Over the course of the experiment a total of five rats were lost from the 50 mg NMU/kg study resulting in a final n=29 for the sedentary and n=26 for the exercised groups. Both sedentary and exercised rats were maintained in the same room on a twelve hour light dark cycle and fed laboratory rodent diet 5001 ad libitum. The rats were exercised for four weeks from 21 days of age to 50 days of age according to the protocol for the MS group in chapter 3 (Table 3.1).

5.2.2 Nitrosomethylurea (NMU) administration

NMU was administered as described in Thompson and Adlakha (1991). Briefly, NMU was purchased from Ash Stevens and dissolved in 0.9% NaCl solution pH 4 (acidified with acetic acid) to a concentration of 14mg/ml. The carcinogen was used within an hour of preparation. At 50 days of age, twenty four hours after the last bout of exercise, the rats were given injections of 37.5 or 50 mg NMU/kg body weight ip. Rats were palpated two times per week for detection of mammary tumors. The experiment was terminated twenty four weeks following carcinogen administration.

5.2.3 Necropsy

The rats were euthanized with gaseous carbon dioxide and skinned. The location of all tumors were noted and compared to palpation records. Tumors were removed and fixed in methacarn. Tissues were then processed for routine histological evaluation. Paraffin sections (5 micron) were prepared and stained with hematoxylin and eosin. Mammary

tumors were classified histologically as recommended by Young and Hallowes (1973). Those tumors which were confirmed to be carcinomas were used to determine cancer incidence, cancer latency and the average number of cancers per animal in each treatment group.

5.2.4 Food intake

For the rats injected with 37.5 mg NMU/kg, food intake was monitored twice a week by measuring the amount placed in the cage minus the amount left at changeover.

5.2.5 Statistical Analysis

Tumor incidence was analyzed by the method of Peto (18), while multiplicity was analyzed with the Wilcoxon Rank sums test. Latency was analyzed with the SAS lifetest. Growth rate, tumor weight and food intake were analyzed by the Student t test.

5.3 RESULTS

The rats in the exercise group were trained from 21 days of age to 50 days of age. At the end of the training period (50 days of age) there was no significant difference in weight between exercised and sedentary rats (Figure 5.1).

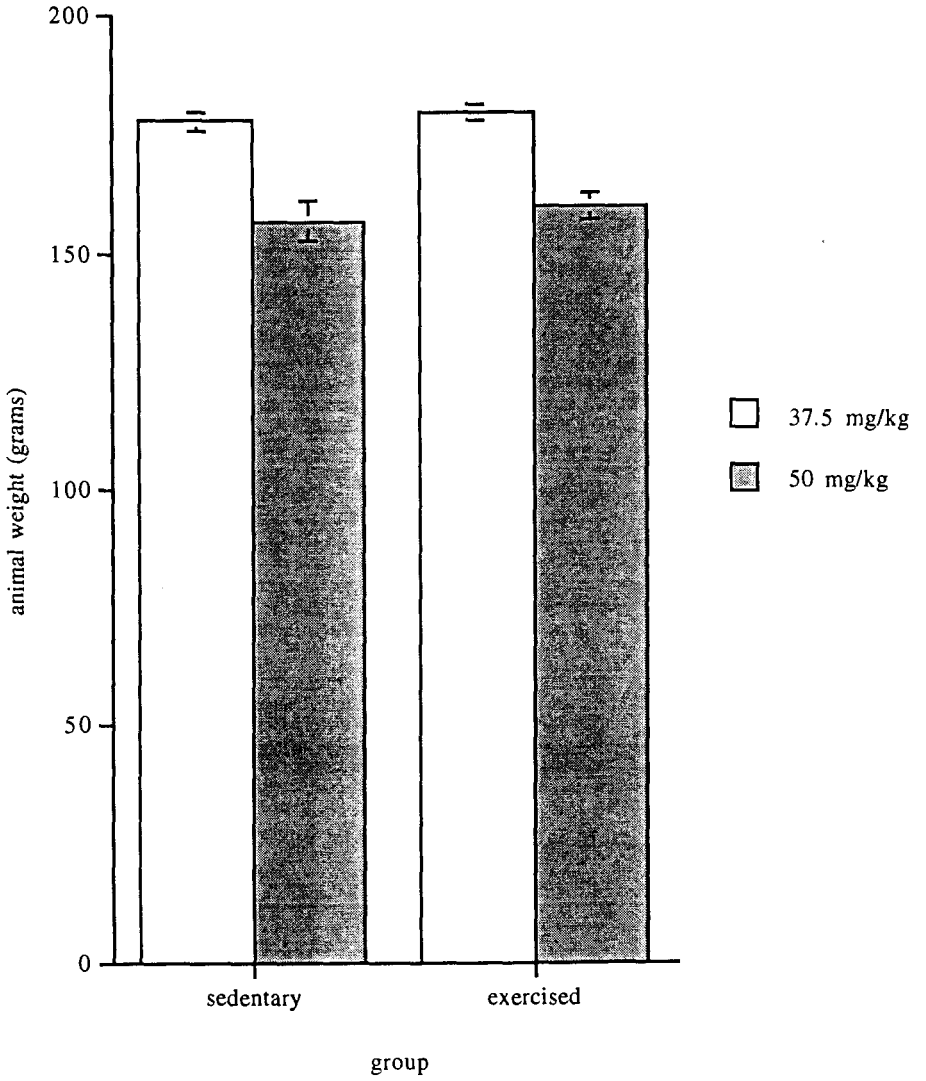


Figure 5.1. Animal weights after exercise training from 21 days of age until 50 days of age and prior to injection of either 37.5 or 50 mg NMU/kg.

An injection of 50 mg NMU/kg resulted in a total (palpated and nonpalpated) of 60 tumors in the sedentary rats and 35 in the exercised rats. The location of each tumor is identified in figures 5.2a and 5.2b respectively. The upper quadrant is defined as mammary glands 1, 2 and 3, while the lower quadrant encompasses mammary glands 4, 5 and 6. For the

sedentary animals, 31 tumors are in the upper quadrant and 29 are in the lower quadrant. In each group, two tumors were identified as benign and were removed from further analysis.

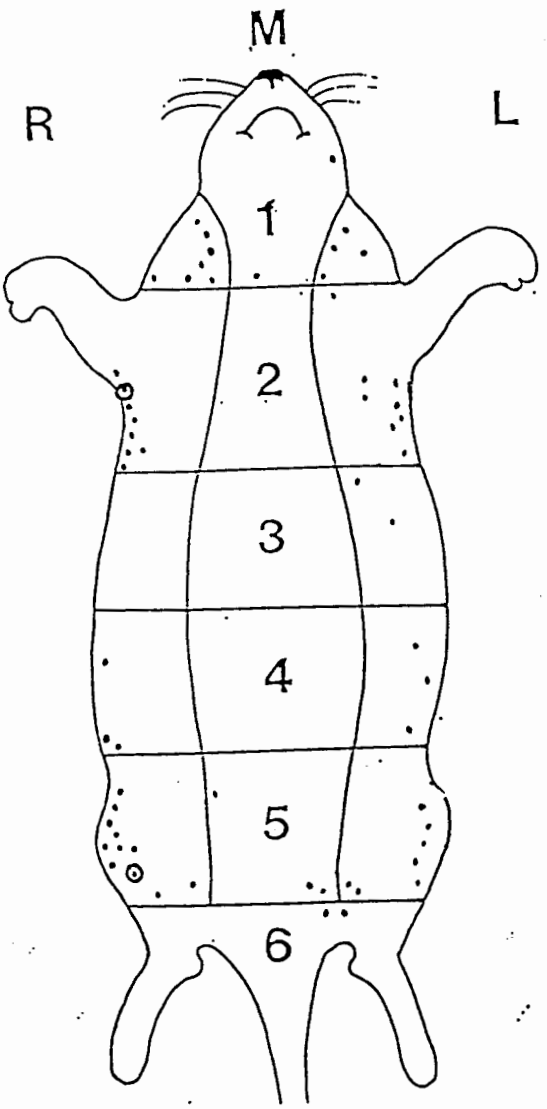


Figure 5.2a. Location of tumors in sedentary rats injected with 50 mg NMU/kg. The circles tumors represent benign tumors.

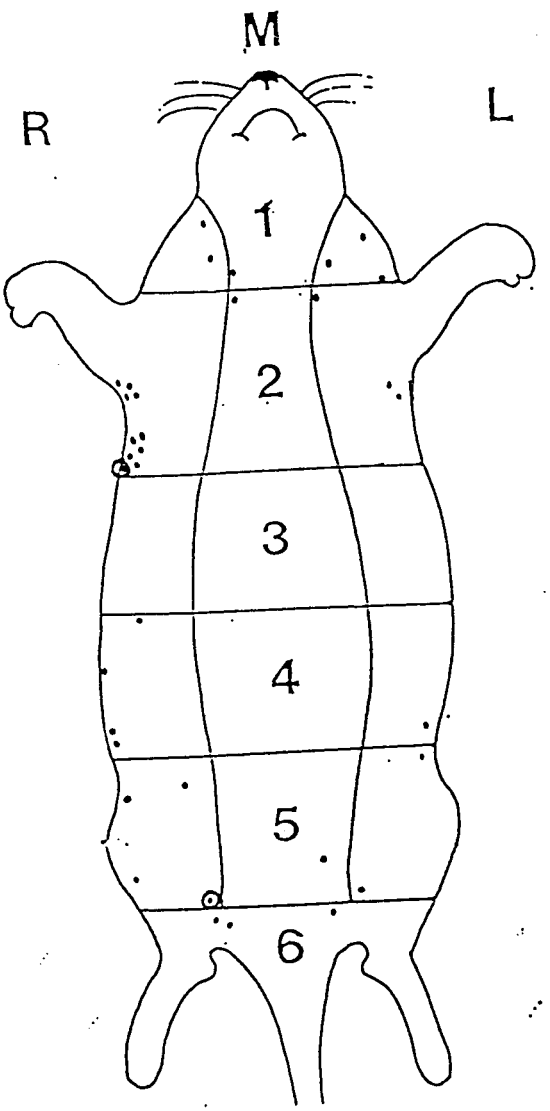


Figure 5.2b. Location of tumors in exercised rats injected with 37.5 mg NMU/kg. The circled tumors represent benign tumors.

As depicted in figure 5.3, there was a significant difference between total number of malignant tumors between the sedentary and exercised groups (58 vs. 33). The total number of malignant tumors is equal to the summation of all the tumors per experimental group. In each group 9 tumors were discovered at necropsy.

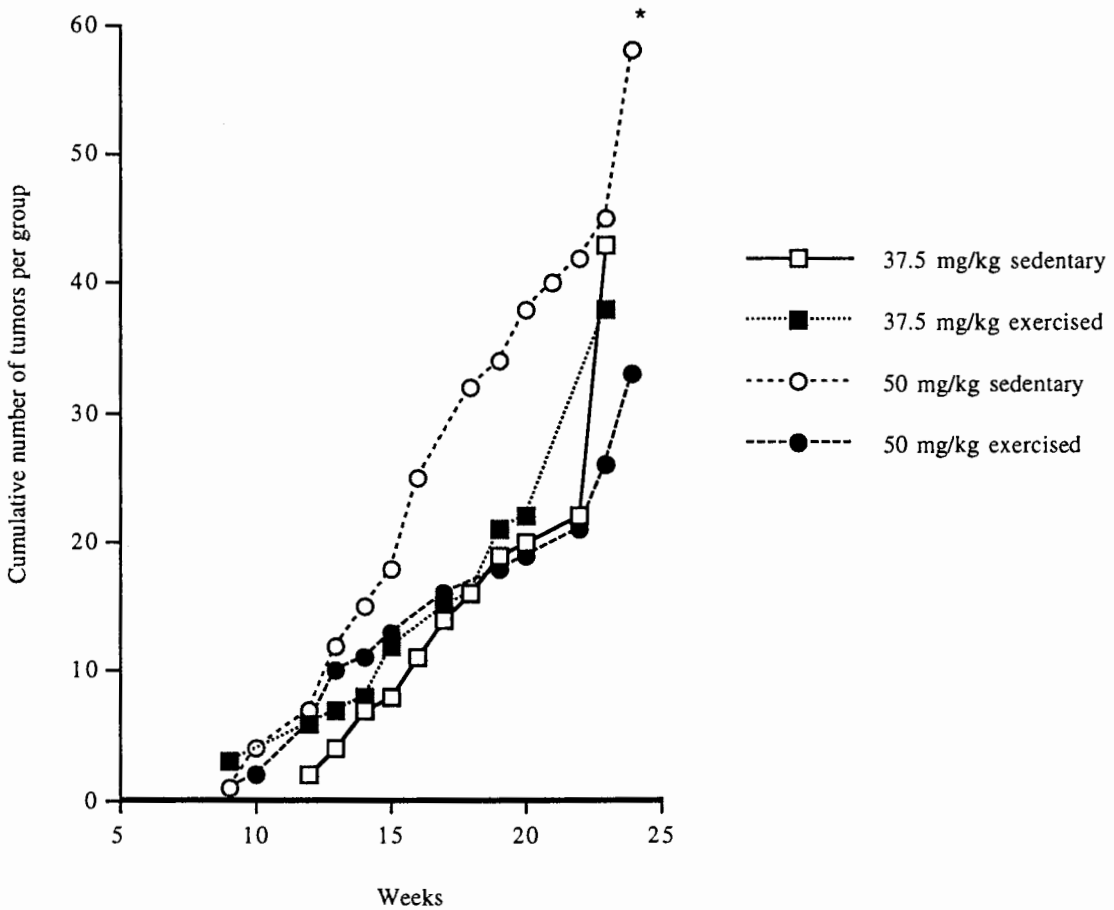


Figure 5.3. Cumulative number of palpable mammary cancers as a function of time post carcinogen administration. Fifty day old rats were given ip. injections of 37.5 or 50 mg NMU/kg. *There is a significant difference in total tumor number between the exercised and sedentary groups for rats injected with 50 mg NMU/kg ($p<.05$).

By lowering the dose of NMU to 37.5 mg NMU/kg, a total of fifty tumors in sedentary and forty three tumors in exercised rats were induced. The location of tumors in animals

injected with 37.5 mg NMU/kg is depicted in figure 5.4a and 5.4b. For the sedentary animals 30 tumors were in the upper quadrant and 20 in the lower quadrant. In the exercised animals, 20 tumors were in the upper quadrant and 23 were in the lower quadrant. Of these tumors, seven were benign in the sedentary group and five were benign in the exercised group.

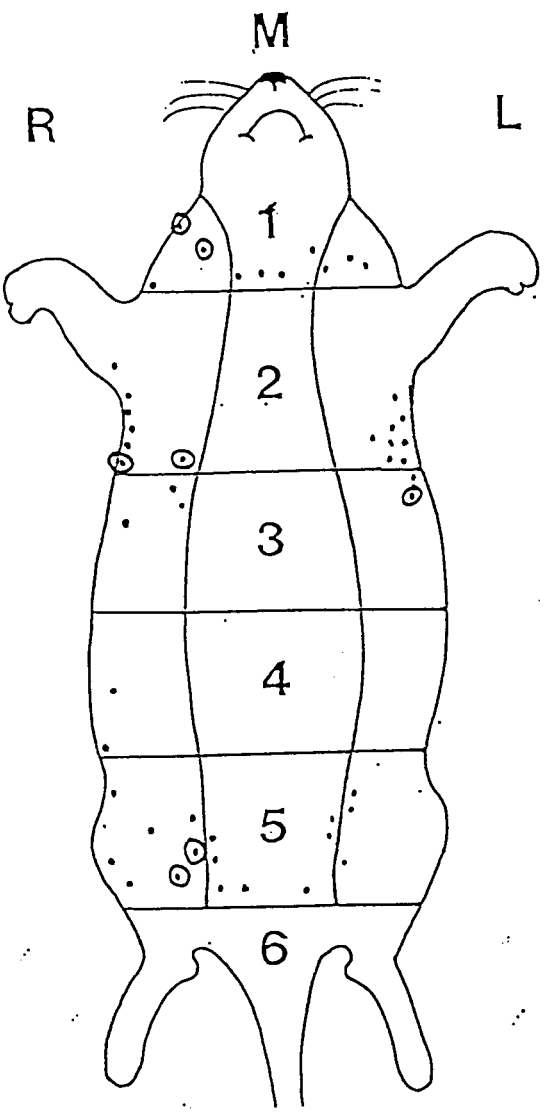


Figure 5.4a. The location of tumors in sedentary rats injected with 37.5 mg NMU/kg. The circled tumors represent benign tumors.

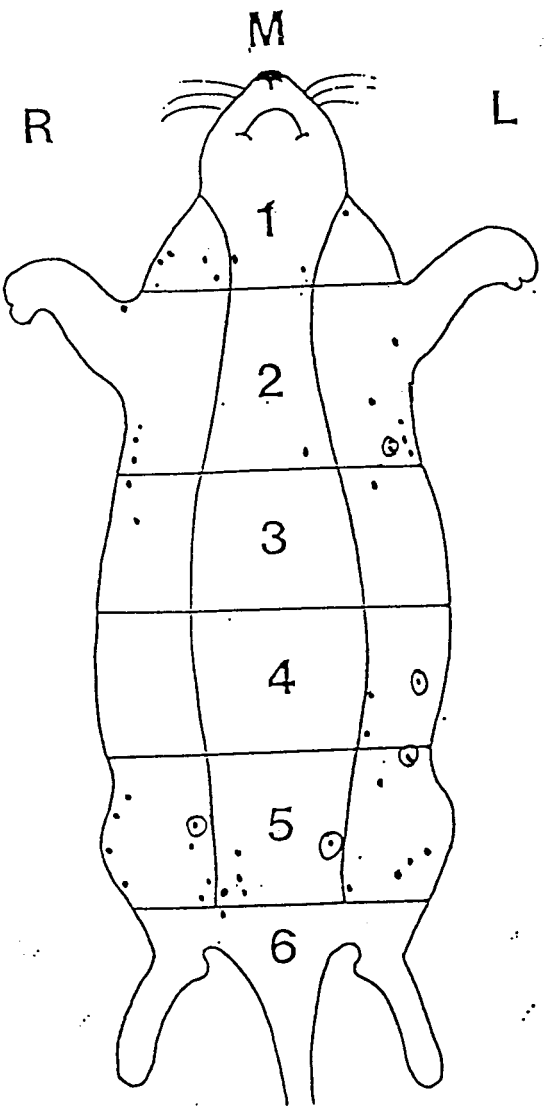


Figure 5.4b. The location of tumors in exercised rats injected with 37.5 mg NMU/kg. The circled tumors represent benign tumors.

As seen in figure 5.3 there was no difference between the total number of malignant tumors in each group (43 vs. 36). For each group twenty two malignant tumors were palpated and

at necropsy in the sedentary group twenty one malignant tumors were discovered and in the exercise group fourteen malignant tumors were identified.

Figures 5.5 reveals the pattern of tumor incidence over the course of the experiment for doses of 50 mg NMU/kg and 37.5 mg NMU/kg. Incidence is defined as the percentage of rats bearing tumors in an experimental group. From time 0 to 15 weeks post carcinogen, the pattern of tumor incidence is nearly identical in the sedentary and exercised rats injected with 50 mg NMU/kg (Figure 5.5). From 15 weeks until termination the two curves diverge slightly, with incidence increasing at a faster rate in the sedentary rats although this was not statistically significant. Seventy five percent of the sedentary and 73% of the exercised rats had malignant tumors. Of these, 69% of the sedentary and 61.5% of the exercised rats had palpable tumors. For rats injected with 37.5 mg NMU/kg the final tumor incidence, although lower in the exercised groups (47.5%), was not significantly different from the sedentary groups (55%). Of these, 35% and 37.5% respectively were palpable.

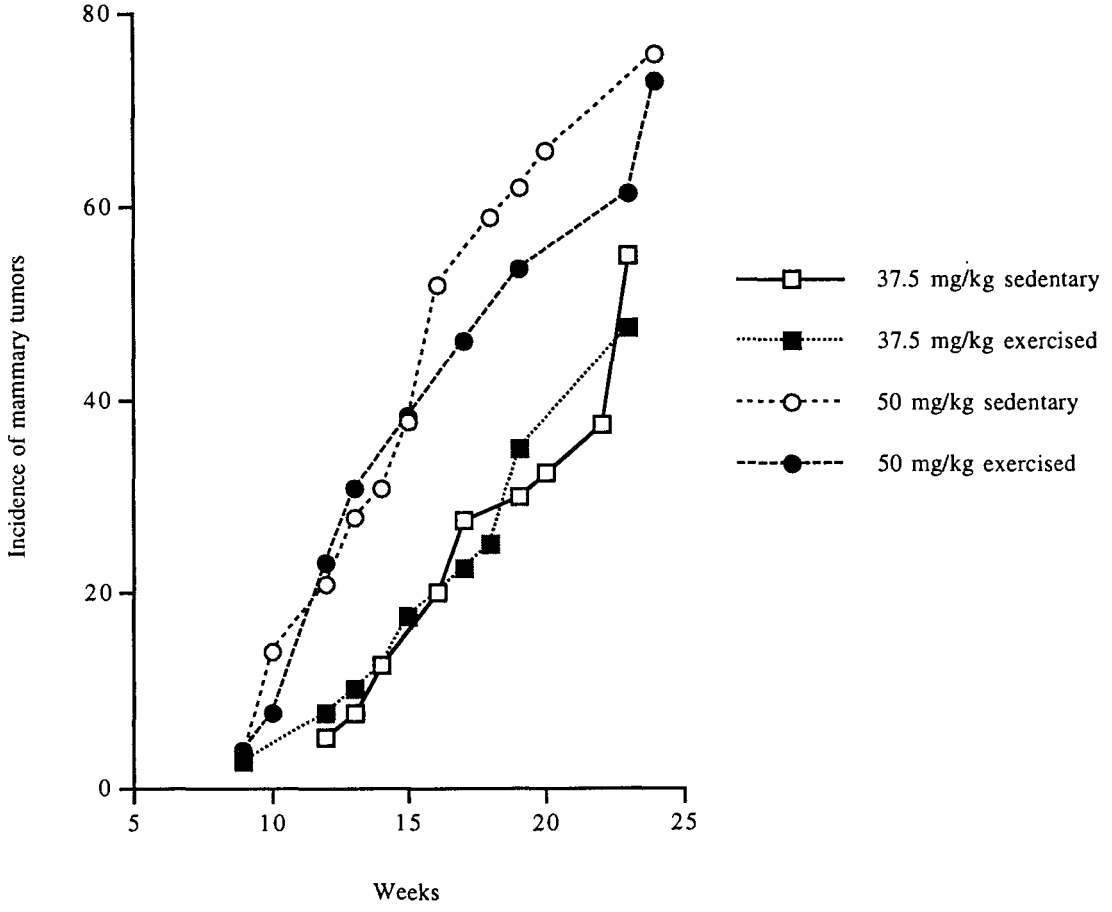


Figure 5.5. Incidence of mammary cancer (expressed as a percentage) as a function of time post carcinogen administration. Fifty day old rats were given ip. injections of either 37.5 or 50 mg NMU/kg body weight. There is no significant difference between exercised and sedentary rats ($p>.05$).

Multiplicity (number of tumors per rat) is determined by dividing the total number of tumors per group by the number of rats bearing tumors. The multiplicity was significantly reduced in the exercised rats at a dose of 50 mg NMU/kg (Table 5.2). The differences between the exercised and sedentary rats in terms of multiplicity was not seen at 37.5 mg NMU/kg.

For rats injected with 50 mg NMU/kg, there was no difference in latency for total malignant tumors (palpated and nonpalpated) between sedentary and exercised rats (128.2± 4.2 days vs. 128.2± 6.3 days respectively) or for palpated malignant tumors (120.5± 4.12 days sedentary vs. 117.2± 6.4 days exercised). In rats injected with 37.5 mg NMU/kg, although the mean latency period for total malignant tumors was 121.4± 5.2 days, this was not significantly different from the sedentary group (129.5± 4.0 days). In addition, there was no significant difference in the latency period for palpated malignant tumors between exercised and sedentary groups (97.8 ± 4.2 vs. 108± 4.2 days respectively).

group	n	final incidence (%)	total tumor number	number of tumors per rat[a]	latency (days)	growth rate (g/day)	tumor weight (grams)
37.5 mg NMU/kg							
sedentary	40	55	43	1.08±0.22	129.5±4.0	0.063±0.022	1.2±0.34
exercised	40	47.5	36	0.90±0.22	121.4±5.2	0.124±0.03*	3.2±0.74*
50 mg NMU/kg							
sedentary	29	75	58	2.0±0.35	128.2±4.2	0.084±0.018	2.6±0.67
exercised	26	73	33*	1.3±0.24*	128.2±6.3	0.070±0.017	2.1±0.65

Table 5.2. The effects of exercise on total tumor parameters in rats injected with 37.5 and 50 mg NMU/kg· The rats were exercised from 21 D.O.A. to 50 D.O.A. and then remained sedentary until the termination of the experiment at 24 weeks post carcinogen administration. [a]This value was calculated using only those rats which had tumors. *There was a significant difference between exercised and sedentary ($p<.05$)

There was no difference in final total tumor weights between sedentary and exercised rats injected with 50 mg NMU per kg body weight (2.6± 0.67 g vs. 2.1± 0.65 g). Nor was there a difference in growth rates between the two groups (0.084± 0.018 g/day vs. 0.070± 0.017 g/day). For the 37.5 mg NMU/kg study there was a significant difference in tumor growth rate between the sedentary (0.063 ± 0.022 g/day) and exercised (0.124 ± 0.03 g/day) groups. As well there was a significant difference in final tumor weight (1.2g ± 0.34 vs. 3.2 ± 0.74 g). Figure 5.6 demonstrates significantly increased food intake for the exercised rats from weeks 2 to 8 inclusive.

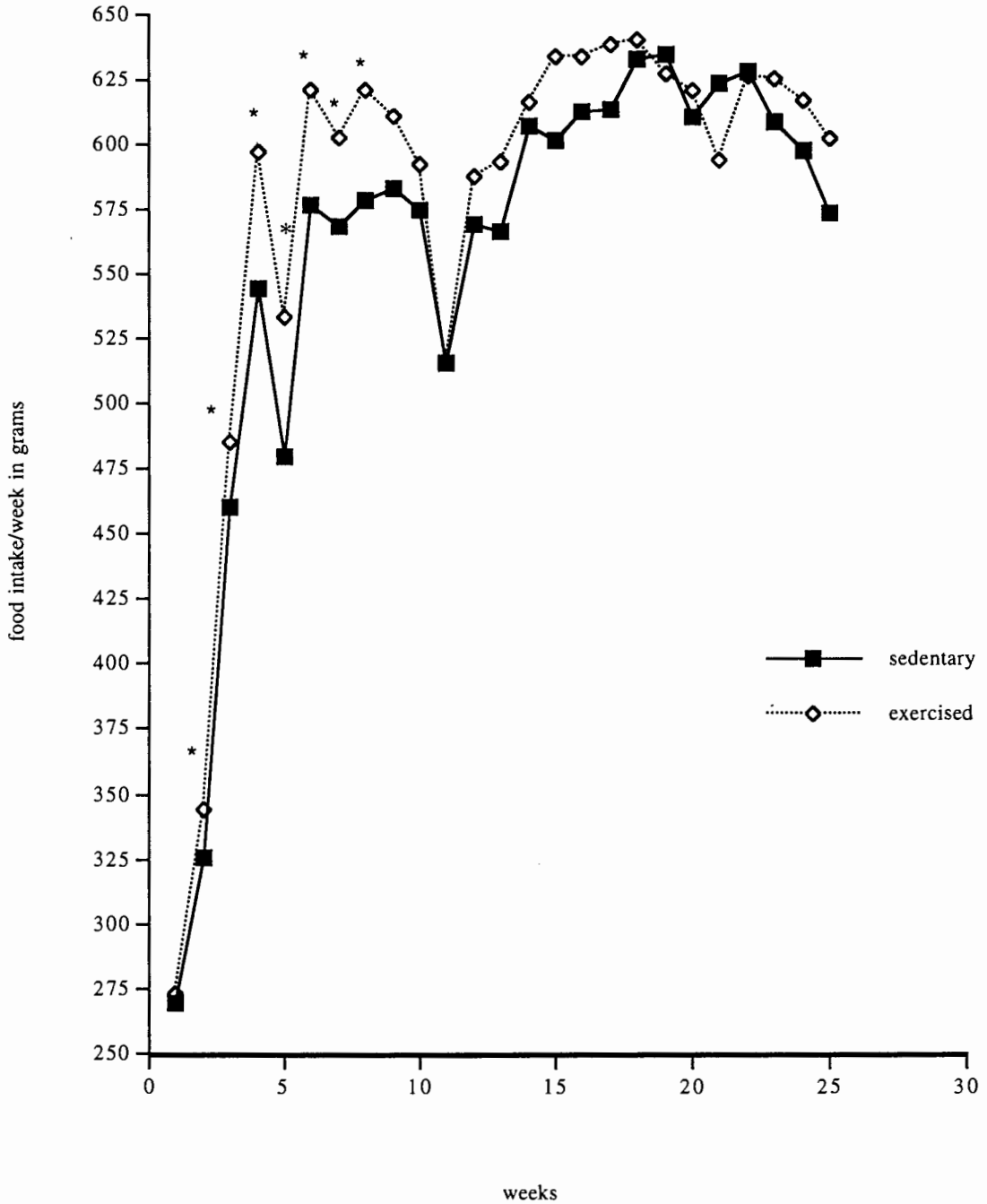


Figure 5.6. Food intake for sedentary and exercised rats injected with 37.5 mg NMU/kg. *There is a significant difference between the two groups from weeks 2 to 8 inclusive ($p<.05$).

5.4 Discussion

Carcinogen induced mammary tumorigenesis is an accepted animal model for studying breast cancer. Because the carcinogen is administered at a certain time, the effects of perturbations both before and after the administration can be assessed. The effects of exercise on mammary tumorigenesis has previously been assessed by utilizing training programs that are initiated two weeks post carcinogen administration corresponding to the promotion phase of tumorigenesis (Cohen et al. 1988; Cohen et al. 1991; Lane et al. 1991; Thompson et al. 1988; Thompson et al. 1989). These studies have shown conflicting results in that exercise can either inhibit or enhance mammary tumorigenesis. Exercise during the promotion phase directly influences the development and growth of a tumor. We wanted to address the question of exercise before carcinogen administration to determine if this perturbation affects the initiation phase and subsequent tumor development.

In this chapter the effects of four weeks of exercise prior to NMU administration on subsequent tumor development was assessed using two doses of NMU, 37.5 and 50 mg NMU/kg. The results of the experiment utilizing 50 mg NMU/kg suggest that an increase in physical activity, achieved through treadmill exercise of moderate intensity for four weeks during peripuberty, can alter the induction of mammary tumorigenesis by NMU in the rat in a positive way. Specifically, those rats exercised prior to carcinogen administration demonstrated reductions in the total number of tumors which corresponds to a decreased number of tumors per animal. There was a trend of lower final tumor incidence and longer latency in the exercised rats; however, these values were not significantly different from the sedentary controls. It appears that exercise is affecting NMU induced mammary tumorigenesis in such a way as to reduce the total number of tumors but not strong enough to be affecting incidence. This effect is not due to changes in mammary gland development

or epithelial cell proliferation which was investigated in chapter 3. Other possibilities include alterations in the immune system and/or residual effects of exercise that affected the promotion phase of tumorigenesis. An NMU dose of 50 mg/kg is routinely used in the rat mammary tumor model as it induces incidences between 70 and 100%. However this dose may be too high for a substantial effect of exercise to occur. Cohen has reported carcinogen dose dependent effects of voluntary exercise using the carcinogen dimethylbenz[a]anthracene (Cohen et al. 1993). With both a low (5 mg/rat) and high (10 mg/rat) dose of DMBA, voluntary activity significantly decreased total tumor number. Latency was significantly lengthened in the low but not high group, while multiplicity was significantly decreased in high but not the low DMBA group (Cohen et al 1993). In order to determine if the dose of 50 mg NMU/kg was too high to see any effects, in the present study the NMU dose was lowered to 37.5 mg/kg. The effect of exercise on multiplicity was lost with the lower dose and as with 50 mg NMU/kg there was no difference in latency or incidence of tumors. One possible explanation is that with more time, exercise may have shown an effect at 37.5 mg NMU/kg. At necropsy 21 tumors were discovered in the sedentary group and only 14 tumors in the exercised group. One limitation of animal carcinogen models is the longer the duration of the study the more animals you lose to tumor burden. Tumors isolated from exercised rats injected with 37.5 mg NMU/kg had a significantly faster growth rate and larger tumor weight than their sedentary counterparts. Two other investigations have looked at the effects of exercise on the initiation phase of carcinogenesis (Yedinak et al. 1988; Thompson 1994). Yedinak et al. (1988) observed a significant decrease in tumor incidence in rats exercised 3 weeks prior to and 3 weeks post DMBA administration, compared to sedentary rats. In order to assess the effects of exercise on the initiation of mammary tumorigenesis, Thompson exercised rats from twenty two days of age until fifty seven days of age, one week past carcinogen administration (Thompson 1994). Thompson (1994) tested both NMU and DMBA. There was no effect of exercise on tumor outcome regardless of the dose of NMU (25 or 50 mg/kg). However with DMBA

exercise reduced the tumor incidence. Our results and those of Yedinak and Thompson suggest that the mechanism through which early exercise affects tumorigenesis is by altering the metabolism of DMBA and that exercise may have a more subtle effect on the NMU model. NMU and DMBA function in two distinct ways: NMU is direct acting, while DMBA requires host activation.

For our study, the rats were exercised during the first 4 weeks of the experiment and then were sedentary for the remainder of the study. The food intake of exercised rats was significantly increased compared to sedentary rats in weeks 2 to 8 inclusive. Female rats typically increase their food intake in response to increased energy expenditure (Hoffman-Goetz and MacDonald 1983). The increased caloric intake may have counterbalanced any effect of exercise at initiation. Additionally the food intake was still elevated four weeks after exercise ceased. This could have had a promoting effect on tumor development. Caloric restriction inhibits the development of rodent mammary tumors (reviewed in Welsch 1992). There is a positive relationship between caloric consumption and rodent mammary gland tumorigenesis (reviewed in Welsh 1992).

The tumor incidence, total number of tumors and tumor multiplicity were higher in the rats injected with 50 mg NMU/kg compared to 37.5 mg NMU/kg in both the sedentary and exercised rats. In the previous chapter, we observed an increased amount of NMU bound to mammary gland epithelial cell DNA when the dose administered to the rat was increased from 37.5 mg NMU/kg to 50 mg NMU/kg. The results from this study suggest that the increased binding of NMU leads to increased induction of tumors.

In general, we detected more tumors in the upper quadrant that in the lower quadrant. In the previous chapter the abdominal glands bound significantly less NMU and contained

significantly less DNA damage. However, in this study, we could not distinguish between tumors that arose from the abdominal and inguinal glands.

In our experiments, particularly with a dose of 37.5 mg NMU/kg exercise had adverse effects on food intake, tumor growth rate and tumor weight, nevertheless, no effect on tumor incidence was observed, suggesting that some mechanism is counteracting these effects. Two possibilities are alteration of the immune system and/or increased capacity for DNA repair.

Tumors from exercised rats injected with 37.5 mg NMU/kg exhibited faster growth rates. Estrogen and insulin-like growth factor-I are believed to be involved in breast cancer. In the next chapter, we assess the presence of estrogen receptor and IGF-I receptor in NMU induced tumors and the relationship of the receptor content to growth rate.

5.5 Appendix

	37.5 mg NMU/kg		50 mg NMU/kg	
	Sedentary	Exercised	Sedentary	Exercised
animals	40	40	29	26
total tumors	50	43	60	35
p	26	26	51	26
np	24	17	9	9
carcinomas	43	36	58	33
p	22	22	49	24
np	21	16	9	9
benign	7	5	2	2
rats-p	17	15	20	16
rats-np	7	5	2	3
total	24	20	22	19
rats with m-p & np	22	19	22	19
% of rats with m-p & np	55%	47.5%	75.9%	73%
rats with m-p	15	14	20	16
% of rats with m-p	37.5%	35%	69%	61.5%
latency-m,b,p	105$\pm$4.3 days	99.3$\pm$4.0 days	120.6$\pm$4.1 days	116.9$\pm$6.0 days
latency-m,p	108$\pm$4.2 days	97.8$\pm$4.2 days	120.5$\pm$4.1 days	117.2$\pm$6.4 days

latency-m,b,p,np	127.5± 4.0 days	120.8± 4.8 days	128.0± 4.2 days	127.3± 6.0 days
latency-m,p,np	129.5± 4.0 days	121.4± 5.2 days	128.2± 4.2 days	128.2± 6.3 days
growth rate-m,b,p,np	0.068± 0.019 g/day	0.115± 0.025 g/day	0.08± 0.018 g/day	0.069± 0.015 g/day
growth rate-m,p	0.063± 0.022 g/day	0.124± 0.029 g/day	0.084± 0.018 g/day	0.070± 0.017 g/day
tumor weight-m,b,p	2.58± 0.59 g	4.9± 1.10 g	2.9± 0.76 g	2.7± 0.74 g
tumor weight-m,p	2.1± 0.48 g	5.2± 1.26 g	3.0± 0.78 g	2.6± 0.80 g
tumor weight-m,b,p,np	1.4± 0.34 g	3.1± 0.74 g	2.53± 0.65 g	2.2± 0.61 g
tumor weight-m,p,np	1.2± 0.28 g	3.2± 0.81 g	2.6± 0.67 g	2.1± 0.65 g

Table 5.5.1. Characteristics of tumors removed from rats injected with either 37.5 or 50 mg NMU/kg. m = malignant tumors; b = benign tumors; p = palpated; np = nonpalpated.

Chapter VI Insulin-like growth factor I receptors and estrogen receptors in NMU induced mammary tumors

6.1 Introduction

IGF-I is a growth factor believed to be involved in breast cancer. IGF-I appears to function in a paracrine manner, it is expressed in stromal cells and acts upon carcinoma cells (Rosen et al. 1991). IGF-I elicits its effects through the membrane bound IGF-I receptor. IGF-I receptor mRNA and protein have been detected in human malignant breast tissue (Pollack et al. 1987; Peyrat et al. 1988; Cullen et al. 1990). A positive link between IGF-I and estrogen receptor protein levels has been suggested (Peyrat et al. 1989; Peyrat and Boneterre 1992). Estrogen receptors have been characterized in NMU tumors with 17β ^{3}H estradiol receptor binding assays (Arafah et al. 1980; Turcot-Lemay and Kelly 1980; Manni et al. 1982). To our knowledge, the IGF-I receptor has not been studied in the NMU induced mammary tumor model. In the previous chapter, exercise significantly increased the tumor growth rate and size of mammary tumors induced by 37.5 mg NMU/kg. In this study we measured the levels of IGF-I and estrogen receptors in normal mammary tissue and tumors from sedentary rats to determine if the amount of receptor was increased in the tumor. We also looked at any differences between sedentary and exercised tumors in terms of receptors and looked at the relationship between receptor content and growth rate, latency and weight of tumors.

6.2 Methods

6.2.1 Receptor isolation

Previously frozen samples of mammary glands or mammary tumors were homogenized in HEPES buffer (100mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),

5 mM EDTA, 1% Triton X-100) containing protease inhibitors (2 mM phenylmethylsulfonylfluoride (PMSF), 1mM bacitracin, 1 mg/ml each of benzamidine, Nα-Tosyl-L-arginine methyl ester, Nα-Benzoyl-L-arginine ethyl ester, 1TIU/ml aprotinin, 60 μM pepstatin and 60 μM leupeptin). Homogenized samples were centrifuged at 20, 000 x g for 10 minutes at 4°C. The supernatant was incubated on ice for 30 minutes with frequent vortexing. The samples were centrifuged at 150, 000 x g for 30 minutes at 4°C. The supernatant was removed and protein concentration determined with Bradford microassay. The samples were lyophilized overnight.

6.2.2 SDS PAGE Electrophoresis and protein transfer

The dried samples were resuspended in 1X urea buffer (2% β-mercaptoethanol, 8 M urea, 1% SDS, 0.1 M TRIS pH 6.8) at a concentration of 2400 μg/ml. The proteins were separated on a 8% SDS PAGE with 312 μg loaded in each lane. The proteins were transferred to PVDF membrane in Tris/glycine buffer with 5% methanol, running at a constant current of 420 mAmps for three hours. The membrane was stained with Ponceau Red to verify that transfer had occurred. The membrane was washed in 1X TBST for fifteen minutes and then blocked in 3% BSA,1X TBST for ninety minutes. The membrane was washed three times with 1X TBST. The membrane was incubated overnight with primary antibody shaking at 4°C (estrogen 1:10,000; IGF-I α 1:5000). The membrane was then washed three times with 1X TBST and incubated for 90 min. with secondary antibody shaking at room temperature (estrogen 1:20,000; IGF-I α 1:30,000). The membrane was washed once with 1X TBST and twice with 1X TBS. The membrane was incubated for one minute with ECL reagents and exposed to hyperfilm for varying times.

6.2.3 Receptor quantification

Relative quantification of the receptor concentration was accomplished by scanning exposed films on an Hewlett Packard scanner and using NIH Image 5.1 to determine area under the peaks.

6.2.4 Statistical Analysis

Receptor levels were analyzed by the Student t test and correlation coefficients.

6.3 Results

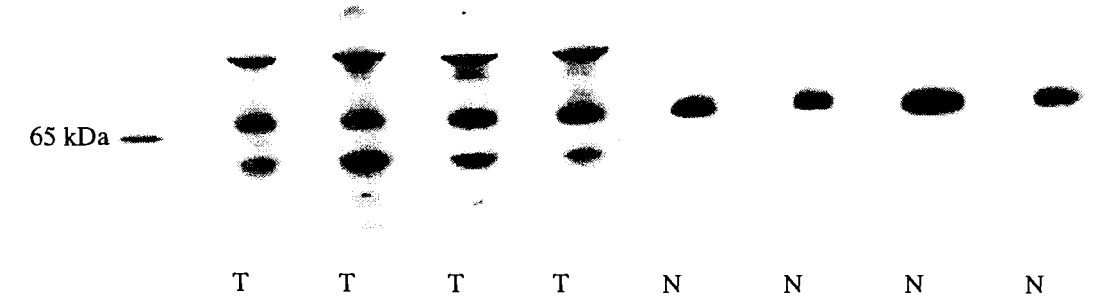
Estrogen and IGF-I receptor levels were measured in NMU induced tumors from sedentary rats and normal mammary glands from age matched rats. The amount of IGF-I receptor α subunit protein was significantly increased in tumors when expressed per μg protein (33%) (Table 6.1). When expressed per mg tissue the tumors expressed 395% more IGF-I α subunit protein. Estrogen receptor content was significantly increased in tumor tissue compared to normal with an increase of 222% when expressed as per μg protein and 485% when expressed per mg tissue (Table 6.1). The total protein yield was also significantly greater from the sedentary tumors compared to the normal mammary gland tissue (Table 6.1). The anti estrogen receptor antibody detected three protein bands in the tumor samples and only one in the normal tissue (Figure 6.1). The molecular weights of the three bands were 81, 75 and 69 kDa.

Receptor levels were measured in tumors from sedentary and exercised rats. Estrogen receptor and IGF-I receptor content expressed per μg protein were not significantly different in tumors from sedentary and exercised rats (Table 6.1). However, when normalized for total protein content per milligram tissue the tumors from exercised rats have significantly less receptor content (Table 6.1). The tumors from sedentary rats had a significantly higher total protein yield (38%).

Group	Total protein yield	IGF-I receptor		Estrogen receptor	
	μg protein per mg tissue	per μg protein	per mg tissue	per μg protein	per mg tissue
normal mammary gland(n=4)	13.5±2.9	7.5±.32	15.1±7.0	6.2±.12	25.2±4.9
sedentary tumor(n=4)	*23.6±1.5	*10.0±.55	*74.8±1.5	*20.0±1.7	*147.4±12.3
sedentary tumor(n=14)	*23.7±2.8	10.5±.57	*75.0±5.7	19.6±1.6	*142.8±14.5
exercised tumor(n=13)	17.2±.97	9.4±.96	50.9±4.6	18.4±1.14	106.8±10.52

Table 6.1. Receptor content in NMU induced tumors from sedentary and exercised rats. The receptor number is expressed in arbitrary units. * Significantly different from control ($p<.05$). Values are expressed as means ± S.E.

A.



B.

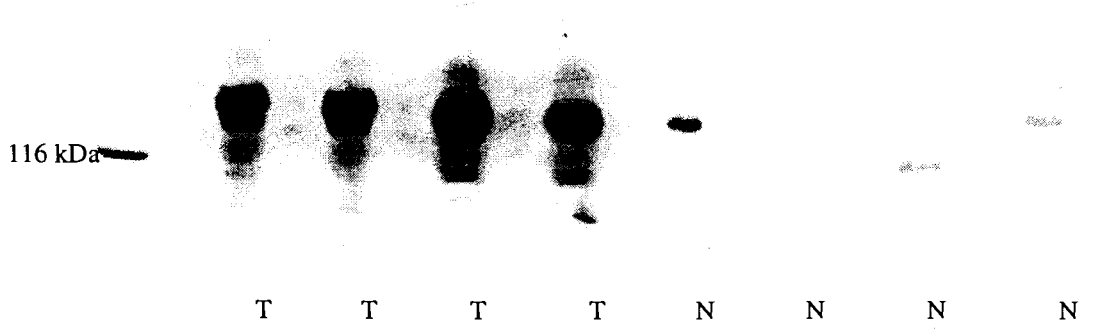


Figure 6.1. Immunoblot determination of A. estrogen receptor content and B. IGF-I receptor content in normal mammary tissue vs. mammary tumor. From left to right, in lane one are molecular weight markers; in lanes two to five are tumor samples; in lanes six to nine are normal mammary gland samples. T= tumor, N= normal

To analyze relationships between receptor number and growth rate, final tumor weight and tumor latency, results from sedentary and exercised tumors were pooled together. There was no significant difference in estrogen receptor number for tumors that had growth rates less than 0.1 grams per day or greater than 0.1 grams per day (Table 6.2). Similarly estrogen receptor number had no influence on latency or final tumor weight (Table 6.2). Correlation coefficients support this data (Table 6.3). However, when tumors from sedentary and exercised rats were analyzed separately, tumors from exercised rats showed a much stronger positive relationship between estrogen receptor number (per μg protein) and growth rate (Table 6.3, figure 6.2). This relationship was also seen with tumor weight.

	Growth rate		Latency		Tumor weight	
	0-0.1 g/day	>0.1 g/day	<100 days	>100 days	< 3 grams	> 3 grams
Estrogen receptor						
per μg protein	7.6±.52	8.9±.58	7.5±.51	8.0±.64	7.8±.54	7.8±.67
per mg tissue	50.3±4.9	54.9±10	49.1±4.5	49.1±6.5	48.4±5.6	50.0±5.7
IGF 1 receptor						
per μg protein	5.8±.40	4.9±50	5.1±.53	6.0±.28	*6.3±.28	4.9±.49
per mg tissue	36.5±2.6	33.5±6.1	32.5±3.3	38.7±3.4	37.1±2.0	33.7±4.6

Table 6.2. Receptor content in relation to growth rate, tumor latency and tumor weight. The results for NMU induced mammary tumors from sedentary and exercised rats were pooled together. The receptor number is expressed in arbitrary units. Values are expressed as means ± S.E.

Per μg protein

Receptor	Growth rate	Latency	Tumor weight
Estrogen			
S and E	0.192	0.064	0.110
sedentary (S)	0.081	0.003	0.075
exercised (E)	0.585	0.110	0.694
IGF-I			
S and E	-0.242	0.241	-0.414
sedentary (S)	-0.350	0.245	-0.166
exercised (E)	-0.314	0.152	-0.265

Per mg tissue

Receptor	Growth rate	Latency	Tumor weight
Estrogen			
S and E	0.175	0.008	0.188
sedentary (S)	0.346	-0.108	-0.229
exercised (E)	-0.360	-0.118	-0.453
IGF-I			
S and E	-0.207	0.234	0.017
sedentary (S)	0.675	0.066	0.314
exercised (E)	-0.338	0.109	-0.252

Table 6.3. Correlation coefficients (r values) for receptor content vs. each of the parameters growth rate, tumor latency and tumor weight.

IGF-I receptor number was significantly lower in tumors greater than 3 grams compared to those that were under 3 grams (Table 6.2). The correlation analysis (r=-.414) supports this data showing an increased tumor size with decreased number of IGF-I receptors (per μg protein) (Table 6.3). This effect was lost when normalizing for the amount of mammary gland tissue. Tumors from sedentary and exercised rats showed a negative correlation with respect to IGF-I receptor number and growth rate when receptor number was expressed per μg protein (figure 6.3). However, when normalized for tumor weight, tumors from sedentary rats revealed a strong positive correlation and tumors from exercised rats a weaker negative correlation (Table 6.3).

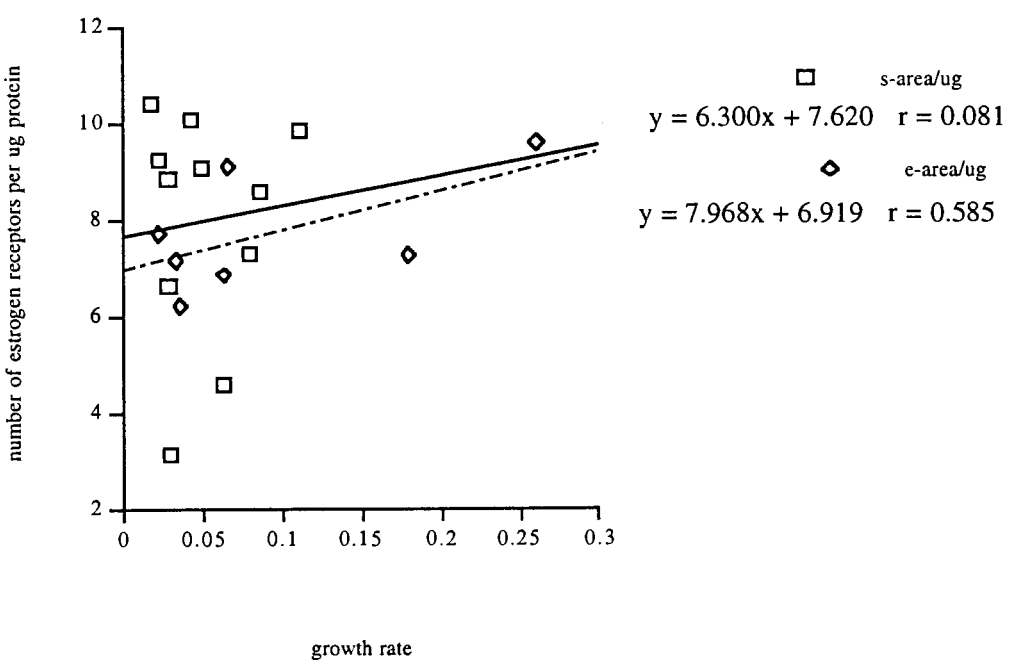


Figure 6.2. Estrogen receptor number vs. growth rate in tumors from sedentary and exercised rats.

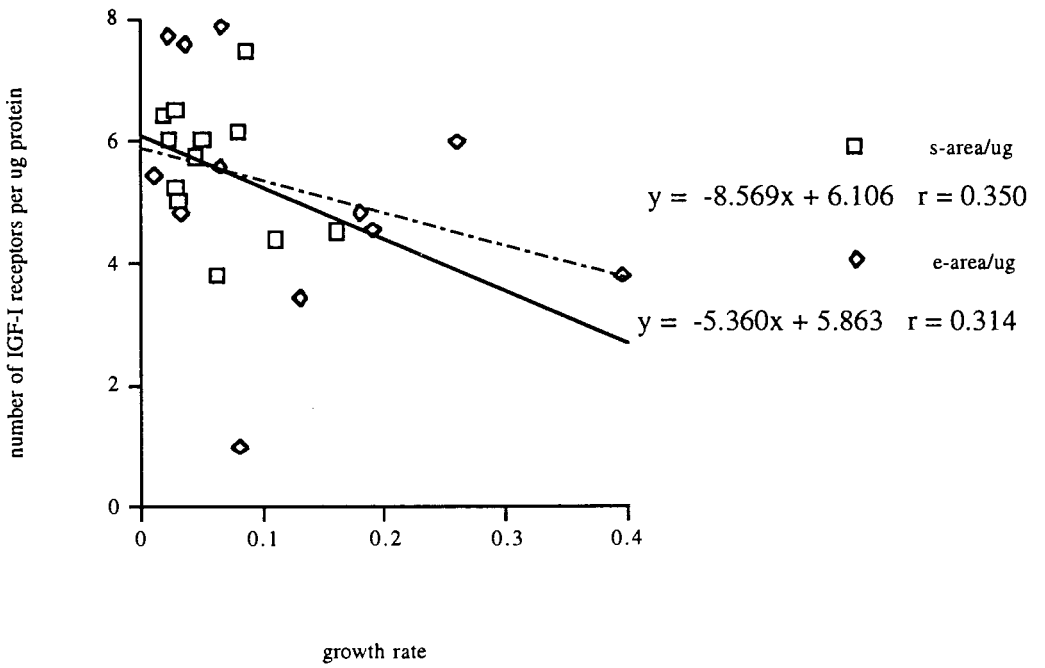


Figure 6.3. IGF-I receptor content vs. growth rate in tumors from sedentary and exercised rats.

6.4 Discussion

To our knowledge, this study is the first to identify IGF-I receptors in NMU induced mammary tumors. It is also the first time western blot analysis has been used to study estrogen receptors in these tumors. The potential presence of three isoforms of the estrogen receptor in tumor tissue agrees with literature on human breast tumors. When compared on a per μg basis, mammary tumors from exercised rats had a stronger correlation between estrogen receptor number and growth rate than sedentary rats, whereas the correlation between IGF-I receptor content and growth rate was negative for both groups.

We compared receptor content in selected sedentary tumors and normal tissue from age matched controls. An antibody directed against the IGF-I α subunit detected a protein with a molecular weight of approximately 130 kDa in both the normal mammary tissue and mammary tumors. The receptor levels were significantly increased in the tumors. One would expect the tumors to have increased IGF-I receptor and estrogen receptor contents compared to normal mammary tissue. The relative amount of epithelial cells is considerably higher in the tumor samples. Of significance in this study is the presence of both IGF-I receptor and estrogen receptor in normal mammary gland tissue and NMU induced tumors. The functionality of the receptor needs to be assessed. The IGF-I receptor has at least three functions: 1) binding of ligand 2) autophosphorylation and 3) downstream signaling. Any one of these may be dysfunctional leading to a receptor that is constitutively turned on or off. Nevertheless, identifying the presence and increased content of the IGF-I receptor in NMU induced tumors is important for further studies utilizing this model to study growth factor involvement in breast cancer.

Breast cancer is a hormone responsive disease. The advantage of using the NMU model is that tumors are hormone responsiveness (Welsch 1985). Estrogen receptors have been characterized in rat mammary NMU tumors using 17β ^{3}H estradiol receptor binding (Arafah et al. 1980; Turcot-Lemay and Kelly 1980; Manni et al. 1982). In competitive binding assays 17β estradiol is ten fold more active than estriol and estrone, whereas testosterone and progesterone are not competitive (Turcot-Lemay and Kelly 1980). The apparent K_D was calculated as 0.42 $\pm$ 0.05 nM (Turcott-Lemay and Kelly 1980). We used western blots to determine the content of estrogen receptors in NMU induced mammary tumors and normal mammary gland tissue. Estrogen receptor levels were upregulated in tumor samples compared to normal mammary tissue. Protein bands were detected at 69, 75 and 81 kDa. These same three isoforms have been identified in human breast tumors

(Puddefoot et al. 1993). They have corresponding pI values of 6.3, 6.8 and 6.6 respectively (Puddefoot et al. 1993). Estrogen receptor variants of 50 and 65 kDa have been identified in the Grunder mouse model system (Sluyser et al. 1993). Breast tumors can be classified as hormone dependent (HD) hormone responsive (HR) or hormone independent (HI). Hormone independence and a low correlation between estrogen receptor content and hormone response in some cases may be explained by the presence of estrogen receptor variants which are either mutated or deleted and are constitutively activated (Sluyser and Mester 1985). Human tumors are identified as estrogen receptor positive or negative by the binding assay. However, this assay does not detect receptors that are present but cannot bind ligand. Their signaling function may still be active. In the Grunder mouse model, when HD tumors became HI, the presence of the 50 kDa protein is increased relative to the 65 kDa (Sluyser et al. 1993). There is no difference in affinity for estradiol in estrogen receptors from HD and HI, suggesting that the 50 kDa has intact binding domain (Sluyser et al. 1993). A number of variant receptor mRNAs have also been identified (reviewed in Auchus and Fuqua 1994).

IGF-I and estrogen receptor levels were similar in tumors derived from sedentary and exercised rats when expressed per μg protein. However, when normalized for total protein content, the exercised group contained significantly less receptor number. This was reflected in the higher protein yield tumors from sedentary rats. The results for estrogen receptor (expressed per μg protein) are in accordance with those of Cohen et al., who used voluntary access to a training wheel (Cohen et al. 1993). Cohen found no difference in receptor number using the 17β ^{3}H estradiol receptor binding assay. In terms of energy balance, exercise training (energy expenditure) is equal to energy restriction. Low fat diets have been shown to reduce mammary tumorigenesis. However, studies looking at high vs.

low fat diets show no difference between the groups in terms of estrogen receptor levels (Mizukami et al. 1992; Kumaki and Noguchi 1990).

Of particular interest is the fact that there are differences in the expression of estrogen receptor and IGF-I receptor in tumors obtained from either sedentary or exercised rats. The exercise program is terminated months before the tumors are removed and analyzed for receptor status. This suggests that the exercise may have an imprinting effect on the phenotype of the tumor. Specifically, the exercise may expose the mammary gland to a different endocrine/paracrine milieu which affects the phenotype of the epithelial cells which are subsequently exposed to a carcinogen.

Because IGF-I and estrogen exert mitogenic effects, we wanted to determine if there was a correlation between receptor levels and tumor parameters such as tumor growth rate, weight and latency. Because there was no difference in receptor concentration between tumors derived from sedentary and exercised rats (per μg protein), the tumors were pooled together.

By comparing the receptor levels in tumors weighing less than 3 grams versus those more than three, there was a significantly greater number of IGF-I receptors in the smaller tumors. This was reflected in the weak negative correlation coefficient (r=-.242), suggesting that as tumor size increased the number of IGF-I receptor decreased. Perhaps there is a move towards IGF-I independence as the tumors grow. IGF-I independence in a subset of rat tumors has been shown (Ethier et al. 1990). For estrogen receptor levels, tumors from exercised rats show a much stronger correlation than sedentary tumors with respect to growth rate and tumor weight. These results, along with the differences in growth rates and tumor weights, suggest the presence of heterogeneic tumors in the sedentary and exercised rats which respond to different factors and/or hormones.

Chapter VII General Discussion and Conclusions

The work in this thesis revealed a number of novel findings regarding 1) the NMU induced mammary tumorigenesis model, 2) exercise prior to carcinogen administration and its subsequent effect on tumor development and 3) IGF-I receptors and estrogen receptors in NMU induced mammary tumors. The NMU model requires the iv. or ip. administration of NMU, an alkylating agent, to the rat. We conducted experiments to determine the distribution and binding patterns of NMU. As the NMU dose administered was increased, the amount of NMU/metabolites circulating in the blood and mammary gland DNA methylation increased. Doubling the amount of NMU administered resulted in 4 times as much NMU bound to DNA. The amount of DNA damage did not increase with increasing doses of NMU. However, increasing the dose from 37.5 mg NMU/kg to 50 mg NMU/kg resulted in an increase in the total number of tumors and an increase in the multiplicity observed. The increases were not as dramatic as those seen with the DNA methylation. Other factors such as DNA repair must play a role in determining tumor outcome. The amount of DNA methylation and the DNA damage induced by NMU was significantly less in abdominal mammary glands compared to thoracic and inguinal mammary glands. Although we can not distinguish between tumors arising from abdominal and inguinal mammary glands, in general we observed more tumors in the thoracic glands than the abdominal and inguinal mammary glands. By identifying the different properties of mammary glands depending on location we can hypothesize why differences are observed in tumor location in the human breast. One possibility is that different regions of the breast are more susceptible to carcinogen binding and DNA damage. The fact that the mammary glands have different properties depending on their location is an important consideration when choosing to study only one gland or set of glands. One example is using thoracic mammary glands for proliferation studies and inguinal glands for whole mounts.

Exercise prior to NMU administration altered mammary tumorigenesis in a somewhat dose dependent manner. Decreased tumor multiplicity was observed in exercised rats injected with 50 mg NMU/kg but not with 37.5 mg NMU/kg. Exercise did not affect tumor incidence or latency period at either dose. Tumor growth rate and final tumor size were significantly increased in exercised rats at a dose of 37.5 mg NMU/kg. Four weeks of moderate intensity exercise during peripuberty had no effect on mammary gland development in terms of altering the differentiation of terminal end buds into alveolar buds. Exercise did increase the proliferation rate of mammary gland lobuloalveolar cells at all intensities and/or durations of the treadmill running. The increased proliferation rate did not correspond to increased DNA damage induced by NMU as there was no difference in nick translation activity in mammary epithelial cells of sedentary and exercised rats. These results support the hypothesis of Russo et al. (1982) that cells in the TEBs and not lobuloalveolar cells are the targets of carcinogen attack.

IGF-I receptors and estrogen receptors were identified in normal and tumor mammary tissue. The receptor content was significantly increased in the tumors suggesting IGF-I receptors and estrogen receptors may play a role in tumorigenesis. Analysis of the estrogen receptor identified 3 potential isoforms. The functionality of the 3 isoforms is unknown. Tumors from sedentary and exercised animals appear to be heterogeneous. The total protein yield is decreased in tumors from exercised animals resulting in decreased IGF-I receptor and estrogen receptor content per mg tissue. Tumors from exercised rats showed a stronger correlation between estrogen receptor content and growth rate. This may explain the faster tumor growth rate observed in exercise animals. Tumors from exercised animals may be more dependent on estrogen receptors and hence estrogen.

There are limitations to the rat NMU induced mammary tumorigenesis model. NMU induced mammary tumors do not metastasize unlike human breast tumors. *Ha-ras* plays an apparent role in the rat NMU model, however it is not significant in human breast cancer. The role of prolactin in more pronounced in the rat model than in human breast cancer. Finally, the lifespan of a rat is condensed into 2 years making extrapolations to humans more difficult.

7.1 Future Directions

A number of interesting new areas of research arise from the results of our studies. We have investigated the distribution and binding patterns of NMU after an ip. injection. Investigators use both iv. and ip. injections to induce mammary tumors with similar success. Similar experiments need to be conducted after iv. injection. NMU is an alkylating agent which transfers a methyl group to the base of a nucleotide. The next logical step would be to assess methylation of various nucleotides at different doses of NMU. The binding of NMU to DNA increased with increasing dose of NMU, however, DNA damage did not increase. The effect of varying the dose of NMU administered on DNA repair capacity needs to be addressed.

We have observed subtle effects of exercise prior to NMU administration on tumor development whereas other investigators who have exercised their animals through the initiation phase have observed a more profound effect (Yedinak 1988; Thompson 1994). A timecourse study implementing exercise at various times over the course of carcinogen administration would determine when exercise is most effective. Because of the apparent differences in the effects of exercise on NMU and DMBA induced tumors observed by Thompson (1994), one should look at the effects of exercise on the metabolism of DMBA.

Increased caloric consumption is associated with increased mammary tumorigenesis (Welsch 1992). Our exercised rats increased their food intake even after the cessation of exercise. It is of interest to determine if the increased food intake counterbalanced effects of increased energy expenditure due to exercise. This question could be addressed by the addition of 2 control groups, an exercise group whose caloric intake is matched with the sedentary ad libitum group, and a sedentary group whose caloric intake is matched with the exercise ad libitum group.

We identified IGF-I receptors in the normal mammary gland and in mammary tumors. The IGF-I receptor has three functions- binding of the ligand, autophosphorylation and triggering downstream events. The functionality of the IGF-I receptor in normal mammary gland and NMU induced tumors needs to be assessed in terms of the forementioned functions. The detection of three protein bands with the anti-estrogen receptor antibody suggest the presence of three isoforms of the estrogen. The question that arises is can all 3 isoforms bind estrogen and act as transcriptional activators?

We observed different growth rates in tumors derived from exercised and sedentary rats. There was a stronger correlation between growth rate and estrogen receptor content in tumors from exercised rats. Additionally, in both groups there was a negative correlation between IGF-I receptor content and growth rate. One needs to assess how the two types of tumors (sedentary vs. exercised) respond to various hormones and/or growth factors. Secondly, the response to hormones/growth factors over the range of growth rates need to be assessed.

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