Effect of Dietary Zinc Intake on N-Methyl-N-Nitrosourea-Induced Mammary Tumorigenesis and Tumor Histology in Sexually Mature Female Sprague-Dawley Rats

by

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ABSTRACT

Breast cancer is influenced by many factors such as reproductive history, hormonal, genetic and dietary factors. Zinc is vital to many physiological processes including DNA synthesis and cell proliferation. Increasing evidence has linked zinc exposure to mammary tumorigenesis. The hypothesis of this project was that dietary zinc levels have an effect on chemical carcinogen-induced mammary tumorigenesis in rats. The overall objective was to determine the effects of dietary zinc intake on the induction of mammary tumorigenesis by N-methyl-N-nitrosourea in sexually mature rats. Twenty one-day old female Sprague-Dawley rats were assigned to one of the following dietary treatment groups: low zinc (3 mg zinc/kg diet), adequate zinc (12 mg zinc/kg diet; ad libitum control) or high zinc (155 mg zinc/kg diet) group. In addition, two pair-fed control groups: pair-fed-to-the-adequate-zinc and pair-fed-to-the-high-zinc groups, were also included. The rats were fed assigned diets from 21-days. At 50-days of age, all rats were injected with N-methyl-N-nitrosourea (50 mg/kg body weight). Starting from six weeks post-N-methyl-N-nitrosourea injection, rats were palpated weekly to monitor the development of mammary tumors. The experiment was terminated 14 weeks post-N-methyl-N-nitrosourea injection. Mammary tumorigenesis was assessed by tumor incidence, numbers, size, burden, multiplicity and latency of both palpable and total tumors. Tumor grade was assessed histologically. Incidence and number of both palpable and total tumors, and multiplicity of total tumors were lower in marginally low zinc rats than in the ad libitum and pair-fed control rats. Multiplicity of palpable tumors, latency, size and burden were lower in the marginally low zinc and pair-fed rats than in the ad libitum control rats. High zinc intake had no effect on all parameters used to assess the N-methyl-N-
nitrosourea-induced rat mammary tumorigenesis. Dietary zinc intake had no effect on tumor histological characteristics as only malignant tumors were observed regardless of dietary zinc intake. In summary, marginally low zinc intake reduced N-methyl-N-nitrosourea-induced mammary tumorigenesis in sexually mature female rats. Some of the inhibitory effects of marginally low zinc intake on N-methyl-N-nitrosourea-induced rat mammary tumorigenesis were due to reduced feed intake associated with low zinc intake, rather than low zinc intake per se. Zinc supplementation, however, had no effect on N-methyl-N-nitrosourea-induced rat mammary tumorigenesis.
# TABLE OF CONTENTS

Abstract .......................................................................................................................... ii

Table of Contents ........................................................................................................ iv

List of Tables ................................................................................................................... vii

List of Figures ................................................................................................................ viii

Acknowledgements ...................................................................................................... ix

CHAPTER 1 ...................................................................................................................... 1

1. INTRODUCTION ...................................................................................................... 1

2. LITERATURE REVIEW ............................................................................................. 3

2.1. Zinc Nutrition and Biological Functions .............................................................. 3

2.2. Development of Mammary Glands ................................................................. 6

2.2.1. Human Breast Development ........................................................................ 6

2.2.2. Rat Mammary Gland Development ........................................................... 7

2.3. Mammary Tumorigenesis ............................................................................... 8

2.3.1. Human Breast Cancer ................................................................................ 8

2.3.2. Chemically Induced Tumorigenesis ............................................................ 12

2.3.3. Rat Mammary Tumorigenesis as a Model for Humans .............................. 15

2.4. Zinc and Mammary Tumorigenesis .............................................................. 18

2.4.1. Plasma/serum zinc concentrations in humans ....................................... 18

2.4.2. Plasma/serum zinc concentrations in animals ....................................... 20

2.4.3. Mammary gland and tumor zinc concentration ..................................... 21

2.4.4. Dietary zinc intake and mammary tumorigenesis ................................. 22

iv
2.5. Pathology of Mammary Tumors ......................................................... 23
2.6. Summary ....................................................................................... 24

3. HYPOTHESIS AND RATIONALE ...................................................... 25

4. OBJECTIVES .................................................................................... 26

5. REFERENCES .................................................................................... 27

CHAPTER 2 ............................................................................................. 35

1. INTRODUCTION .................................................................................. 35

2. MATERIALS AND METHODS ........................................................... 37

2.1 Dietary Treatments and Animals ....................................................... 37
2.2 Induction of Mammary Tumorigenesis ............................................. 38
2.3 Assessment of Zinc Status ............................................................... 39
2.4 Assessment of Mammary Tumorigenesis ......................................... 40
2.5 Classification of Mammary Tumors .................................................. 41
2.6 Statistical Analysis ......................................................................... 42

3. RESULTS ............................................................................................ 43

3.1 Body zinc status ............................................................................. 43
3.2 Influence of dietary zinc intake on the incidence of mammary tumors ........................................ 44
3.3 Influence of dietary zinc intake on the number of mammary tumors ........................................ 45
3.4 Influence of dietary zinc intake on tumor multiplicity, burden, and latency . 46
3.5 Influence of dietary zinc intake on tumor size ................................... 47
3.6 Effects of dietary zinc intake on mammary tumor grade .................... 47

4. DISCUSSION ....................................................................................... 49

4.1 Development of marginal zinc deficiency......................................... 49
4.2 Low dietary zinc intake suppressed MNU-induced mammary tumorigenesis

4.3 Inhibitory effects of reduced feed intake on MNU-induced

4.4 Lack an effect of zinc supplementation on MNU-induced mammary
tumorigenesis

5. REFERENCES

CHAPTER 3

1. MODEL OF RAT MAMMARY TUMORIGENESIS AND HUMAN BREAST CANCER

2. EFFECTS OF DIETARY ZINC INTAKE ON MAMMARY TUMORIGENESIS

3. EFFECTS OF DIETARY ZINC INTAKE ON HISTOLOGICAL CHARACTERISTICS OF MNU-INDUCED IN RAT MAMMARY TUMORS

4. OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

5. REFERENCES

APPENDICES

APPENDIX 1 DIETARY FORMULATIONS

APPENDIX 2 HISTOLOGICAL ORGANIZATION OF N-METHYL-N-NITROSOUREA-INDUCED RAT MAMMARY TUMORS
LIST OF TABLES

Table 2-1. Criteria for diagnosis of benign and malignant tumors ........................................... 55
Table 2-2. Criteria for diagnosis of papillary carcinoma Grade I and Grade II ....................... 56
Table 2-3. Effects of dietary Zn intake on body zinc status in rats injected with
N-methyl-N-nitrosourea ........................................................................................................... 57
Table 2-4. Effect of dietary zinc intake on the induction of mammary tumors by
N-methyl-N-nitrosourea ........................................................................................................... 58
Table 2-5. Effect of dietary zinc intake on the size of N-methyl-N-nitrosourea-induced
mammary tumors .................................................................................................................. 60
Table 2-6. Effect of dietary zinc intake on the type of N-methyl-N-nitrosourea-induced
mammary tumors .................................................................................................................. 61
Table 2-7. Effect of dietary zinc intake on tumor grade of N-methyl-N-nitrosourea-
induced mammary tumors ................................................................................................. 62
Table A-1. Zinc-free mineral premix ....................................................................................... 85
Table A-2. Vitamin Premix ..................................................................................................... 86
Table A-3. Zinc premix for dietary treatments ......................................................................... 87
Table A-4. Modified AIN-93G egg white based diet ............................................................... 88
LIST OF FIGURES

Figure 1-1. General scheme describing the mechanism of chemical-induced tumorigenesis ................................................................. 14

Figure 2-1. Effect of dietary zinc intake on the cumulative incidence of palpable mammary tumor in N-methyl-N-nitrosourea treated rats ....................... 63

Figure 2-2. Effect of dietary zinc intake on the cumulative number of palpable mammary tumors in N-methyl-N-nitrososurea treated rats .......................... 65

Figure 2-3. Grade 2 papillary carcinoma ......................................................... 67

Figure 2-4. Transition between Grade 1 papillary carcinoma with cribiform pattern and fibroadenoma ................................................................. 69

Figure A-1. Normal and Grade 1 papillary carcinoma ........................................... 90

Figure A-2. Grade 2 papillary carcinoma with thickened epithelial cell layer and absence of a fibrovascular core, pleomorphic nuclei, prominent nucleoli and frequent mitotic figures ......................................................... 92

Figure A-3. Comedo, cribiform and ductal carcinoma ........................................... 94

Figure A-4. Grade 2 papillary carcinoma with cribiform areas .............................. 96

Figure A-5. High grade tumor with invasion ....................................................... 98

Figure A-6. Low grade in situ and invasive carcinoma ......................................... 100
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CHAPTER 1

1. INTRODUCTION

Breast cancer is the most common type of cancer in Canadian women and is one of the leading causes of death in women with cancer in Canada. According to the National Cancer Institute of Canada, 21,100 women are expected to develop breast cancer and deaths due to breast cancer are estimated to be 5,300 in 2003. The ratio of deaths to new cases of breast cancer is 25%. The incidence of breast cancer has risen steadily over the past decade, possibly due to an increase in mammographic examinations and to reproductive histories (National Cancer Institute of Canada, 2003). The high incidence and mortality rates cause enormous emotional and physical stress to women suffering from this disease and increase the financial cost to the ailing health care system. Although the etiology of this disease is not yet established, it has been demonstrated that the development of breast cancer is influenced by many factors such as ionizing radiation, socioeconomic status and endocrinologic, familial or genetic factors and diet (Russo and Russo, 1987).

It is estimated that up to 50% of breast cancer deaths are attributed to dietary influences (Ames et al., 1995). Diet can have an impact on genetics, hormone production and reproductive history, all of which are among the main risk factors for breast cancer. However, the dietary factors that influence the risk of breast cancer are still unknown. Zinc, an essential trace mineral, plays an important role in growth, immune function and cognitive development (Vallee and Falchuk, 1993). Zinc is known to be involved in more than 300 metalloenzymes and over 100 transcription factors involved in gene expression and regulation. Furthermore, it has been well established that zinc depletion suppresses cell
proliferation and growth while zinc repletion supports cell proliferation and growth. Clearly, an optimum zinc status is essential or maintaining normal cellular functions.

In summary, diet is considered as a risk factor for breast cancer and increasing evidence obtained from human and animal studies suggests that zinc has an important role in mammary tumorigenesis (Chakravarty et al., 1986; Philcox et al., 1994; Woo and Xu, 2001). Thus, determining the role of dietary zinc in mammary tumorigenesis is important in understanding how zinc influences the risk of mammary tumorigenesis. My thesis research project was designed to determine the effects of dietary zinc intake on the induction of mammary tumorigenesis in rats. The findings obtained are an initial step towards elucidating the role of zinc in mammary tumorigenesis.
2. LITERATURE REVIEW

2.1. Zinc Nutrition and Biological Functions

Zinc, a trace element, is essential for maintaining normal life in both animals and humans. This was first shown in the early 1930s when researchers found that zinc was required for growth in rats and mice (Jackson, 1989). The total zinc content in an adult human (70 kg) is approximately 1.5 to 2.0 grams (Gibson, 1990). Zinc is present in the body as divalent cations (Zn$^{2+}$) that are primarily bound to macromolecules such as proteins. Zinc is found in all organs, tissues, body fluids, and secretions, but over 80% of zinc is found in skeletal muscle and bone as they make up a large part of the body (Jackson, 1989). Intracellular zinc accounts for over 95% of total zinc in the body, while extracellular zinc is very low. The Recommended Daily Allowance of zinc for males and females is 8 and 11 mg/day, respectively, (Institute of Medicine, 2001). Since most zinc is associated with protein in food, the richest dietary sources of zinc are foods high in protein such as red meat, meat products and seafood. Other good sources of dietary zinc include poultry, dairy products, and whole grains. However, due to the presence of fibre and phytate, fruits and vegetables not only are poor sources of dietary zinc, but also inhibit zinc digestion and absorption.

The physical and chemical properties of zinc and its stable association with other macromolecules make it highly adaptable in meeting the needs of proteins and enzymes that carry out diverse biological functions (Vallee and Falchuk, 1993). Since zinc is found in all cells of the body and is the second most abundant trace element, it has many important functions, three of which are catalytic, structural and regulatory (Cousins, 1996). The catalytic role of zinc can be seen in enzyme catalysis where the removal of zinc renders the
enzyme to be inactive. The structural role of zinc can be found in proteins such as transcription factors where zinc is required to maintain the proper shape of these proteins for their functions. Due to the coiling and twisting nature of the structure while zinc binds to certain amino acids, these structures are called zinc-finger proteins because of their resemblance to fingers (Coleman, 1992). The regulatory role of zinc is perhaps best demonstrated by its effects on apoptosis. At lower concentrations, zinc induces apoptosis while at high concentrations zinc inhibits apoptosis (Sunderman, 1995).

Zinc is present in over 300 enzymes and these metalloenzymes affect most major metabolic processes (Vallee and Falchuk, 1993). Some of the zinc metalloenzymes and metalloproteins are recognized to participate in growth and development. For example, zinc is required for DNA polymerase and thymidine kinase activity, two enzymes that are important for DNA synthesis. Zinc also plays a role in RNA synthesis by enabling RNA polymerase to recognize specific promoter regions and to initiate RNA synthesis. Because it is required for the function of enzymes involved in DNA replication and the transcription factors, zinc is clearly important in gene expression, protein synthesis and cell division.

Inadequate zinc consumption either through inadequate intake or through impaired absorption especially in early life will retard growth and development (Ploysangam et al., 1997). It has been proposed to be due to the requirement of zinc for the enzymes necessary for DNA and RNA synthesis and cell division. Therefore, zinc deficiency has severe effects in organs that have rapid cell division such as the intestines, skin and gonads (Clegg et al., 1989). Since there is no known storage site in the body for zinc, signs of zinc deficiency appear quickly. Two early symptoms of zinc deficiency are reduced feed intake and growth retardation. Although the mechanisms are presently unclear, it is generally accepted that in
order to adapt to lowered zinc intake, feed intake is reduced while tissue catabolism occurs to release zinc from tissues, especially muscle tissues. The zinc released is then used for essential metabolic processes such as protein synthesis, which is needed for survival. Other symptoms of zinc deficiency include lowered plasma zinc concentration, abnormal immune function, delayed sexual maturation, hypogeusia, dermatological lesions such as alopecia and acrodermatitis (Clegg et al., 1989).

Studies show that zinc deficiency in animals and in cultured cells can induce apoptosis. Apoptosis, a gene-directed cell death, is characterized by chromatin condensation, reduced cell volume, blebbing of the cell membrane and fragmentation of the cell. DNA is also cleaved into approximately 180 base-pairs (Sunderman, 1995; Slingerland and Tannock, 1998). There presently are very limited in vivo studies investigating the effect of zinc deficiency and apoptosis. Elmes (1977) first showed an increased number of apoptotic bodies in the crypt region of the small intestine of zinc deficient rats compared to control rats. More recently, Jankowski-Hennig and colleagues (2000) reported that low maternal zinc intake resulted in increased apoptosis in rat embryos. These studies suggest that zinc deficiency can induce apoptosis in vivo. In cultured cells, zinc deficiency induced by the membrane permeable metal ion chelator N, N, N', N'-trakis (2-pyridylmethyl) ethylenediamine (TPEN) induces apoptosis (Zalewski, et al., 1991; Chai et al., 2000; Nakatani et al., 2000). Zinc supplementation in cell culture medium prevents cells from apoptosis induced by apoptotic inducers (Perry et al., 1997; Aiuchi et al., 1998; Fukamachi et al., 1998; Leccia et al., 1999). Although the mechanisms involved are not clear, the protective mechanism of zinc on apoptosis appears to be through its influence on Bcl-2, an antiapoptotic protein, and Bax, a proapoptotic protein (Fukamachi et al., 1998). When zinc
was supplemented in the cell culture medium, the levels of Bax protein decreased significantly resulting in an increase of the Bcl-2/Bax ratio, thus reducing apoptosis. Another possible mechanism is through its influence on the regulation of caspase-3, a member of the caspase family involved in apoptosis. Zinc supplementation to the culture medium suppresses the activation of caspase-3 from its precursor resulting in inhibition of apoptosis (Perry et al., 1997; Aiuchi et al., 1998). All these studies provide evidence suggesting that zinc can regulate apoptosis. Zinc deficiency due to low dietary intake in animals or chelation in cultured cells can induce apoptosis while zinc supplementation prevents apoptosis.

During excessive zinc intake, the body responds by increasing excretion and decreasing absorption. Mice given 3,000 mg zinc/kg diet have no significant pathological signs. However at an intake level of 30,000 mg zinc/kg diet there were gastrointestinal lesions such as ulcers and increased mucus secretion (Walsh et al., 1994). In rats given 100 to 400 mg zinc/kg diet, high intestinal, plasma and tissue zinc levels will be observed for about two weeks (Panemangalore and Bebe, 1996). However, these elevated levels of tissue zinc will decrease to the levels found in the controls because of the homeostatic mechanisms.

2.2. Development of Mammary Glands

2.2.1. Human Breast Development

The human breast consists of 6-10 primary duct systems, which are subdivided into lobules (Russo et al., 1990; Russo and Russo, 1996). The development of human breasts during childhood consists mainly of general growth. Before puberty, these lobules end blindly. As puberty approaches, the lobules proliferate distally and give rise to secondary epithelium-lined ducts in response to the release of estrogen and progesterone. Over the
course of one to two years, the mammary gland tissue increases because of the growth and division of these primary and secondary ducts. Successive branching of these ducts ends in a bulbous formation called terminal ducts (TD), which give rise to clusters of 6-11 alveolar buds (AB). The TD and AB together form a Type 1 lobule, also called a terminal ductule lobular unit (TDLU), the basic functional units of human breasts. TDLUs are the most undifferentiated lobule type and they evolve into Type 2 lobules, which have approximately 47 AB.

Differentiation within mammary glands takes place over many years and is only completed upon pregnancy under the influence of dramatically increased levels of estrogen and progesterone during pregnancy. At this time the intensity of budding and the degree of lobule formation are at the highest when Type 1 and Type 2 lobules further evolve into Type 3 lobules, which have an average of 80 AB. Type 4 lobules are only present during lactation and revert to Type 3 after weaning. In the mammary gland of nulliparous women the predominant lobules are Type 1 (50-60%) and Type 2 (30-35%) with Type 3 lobules making up only 5-10%. In contrast, in premenopausal parous women Type 3 lobules account for 80-100% of the total lobular component.

2.2.2. Rat Mammary Gland Development

There are six pairs of mammary glands in rats: one in the cervical, two in the thoracic, one in the abdominal and two in the inguinal regions. At birth, these six pairs of mammary glands consist of a single primary lactiferous duct, which branch into three to five secondary ducts during the first week of life. These ducts end in terminal end buds (TEB), which are analogous to the TDLU in humans. In the second and third weeks of life, the secondary
ducts will further branch into third, fourth, fifth and possibly sixth generations of ducts. This will result in a very high density of TEB and reach a maximum at 21 days of age. The TEB begin to differentiate into three to five smaller AB from 21 to 62 days of age. As puberty begins between 35-42 days of age hormone release stimulates the AB to differentiate into lobules, which consist of 10-12 alveoli. The progressive differentiation of TEB to AB is accentuated by successive estrous cycles. In rats, the TEB closest to the nipple are the first to differentiate into AB whereas in humans there is no such “gradient”. As in humans, increased hormone levels during pregnancy increase the rate of differentiation of the mammary glands in rats. The hormones produced during this period of time stimulate cell proliferation. The number of TEB is decreased as they differentiate into AB and AB into lobules. The lobules increase significantly in size and in the number of component alveoli (Russo et al., 1982).

Unlike in humans where the ductal structures grow mainly along connective tissue, the ducts commonly grow into the surrounding fat pad in rats. As well, there is a correlation between mammary gland development and age in rats whereas in humans this correlation is not present (Russo et al., 1982; Russo et al., 1990).

2.3. Mammary Tumorigenesis

2.3.1. Human Breast Cancer

The risk of breast cancer is influenced by many factors such as reproductive, hormonal and dietary, and genetic factors. Although the etiology is presently unknown, it is generally believed that breast cancer is originated in the TD. Women who were pregnant at an early age have a lower risk of developing breast cancer when compared to nulliparous
women. This could possibly be because nulliparous women have mainly Type 1 and 2 lobules, which have a higher rate of cell proliferation than the more differentiated Type 3 lobules in parous women. Based on autopsies performed in women with and without breast cancer, TDLU give rise to ductal carcinoma *in situ*, Type 2 lobules give rise to lobular carcinoma *in situ* and Type 3 and 4 lobules give rise to benign breast lesions (Russo *et al*., 1990; Russo and Russo, 1996).

Age at menarche is associated with breast cancer risk. Early menarche and late menopause will increase breast cancer risk because exposure to ovarian hormones is prolonged. For each year that menarche is delayed, there is a 20% decrease in breast cancer risk. Parity and age at first birth can also influence breast cancer. Nulliparous women have a 1.4 greater chance of developing breast cancer compared to parous women. Women who have their first full term pregnancy after age 30 are two to five times more likely to develop breast cancer than women who have their first full term pregnancy before the age of 18-19 (Harris *et al*., 1997). During pregnancy, under the influence of hormones, breast epithelial cells proliferate and differentiate thus decreasing the number of undifferentiated and consequently susceptible cells (Russo *et al*., 1990). However, pregnancy is believed to cause a short-term risk as the cells are proliferating rapidly but it has long-term protection (Key, 1995).

In women with a family history of breast cancer, the risk of developing breast cancer is increased. For example, if the mother or sister of an individual has the disease, her risk of developing breast cancer is increased by one and a half to three times (Harris *et al*., 1997). Mutations in genes such as BCRA-1, BCRA-2 and p53 have been found to increase the risk of breast cancer. Mutations in the BCRA-1 gene located on chromosome 17q21 and the
BRCA-2 gene located on chromosome 13 are associated with a 49% and 28% increase in the risk of developing breast cancer by the age of 50, respectively (Bennett, 1999). Over the lifetime, mutations in the BCRA-1 and BCRA-2 are associated with an 85% increase in the risk of breast cancer. The proteins from these genes along with RAD51 play a role in DNA repair. The p53 tumor suppressor gene is located on chromosome 17p13 (Dickson and Lippman, 1997). In the event of DNA damage, the p53 protein can slow down cell growth and induce DNA repair. However, if the damage is too severe then cell death is triggered. A mutation in this gene alters its confirmation and thus it loses its tumor suppressing ability. In Li-Fraumeni syndrome patients who carry germline mutations in the p53 gene has a high incidence of breast cancer.

There is human and animal research on the possible link between dietary fat, fibre and vitamins and breast cancer risk. According to Canada’s Food Guide to Healthy Eating, a healthy diet includes five to ten servings of fruits and vegetables, and five to twelve servings of grain products and low fat. High intakes of fruits, vegetables, whole grains, low fat dairy and lean meats and poultry are associated with a decreased risk of breast cancer (Gandini et al., 2000; Kant et al., 2000). A healthy diet provides vitamins and minerals that have a positive effect on DNA synthesis and repair, and the lowered fat intake may reduce the risks associated with fat.

It is estimated that up to 50% of breast cancer deaths in the United States can be attributed to dietary factors (Ames et al., 1995). Observations on national fat intake and the incidence of breast cancer in different countries showed that women with breast cancer consumed more fat than the controls. The mechanism by which dietary fat exerts its effect on breast cancer may be through its effect on lipid signaling pathways and sex hormone
levels. Clinical studies show that high fat intake can increase levels of circulating free estrogen, which is associated with high breast cancer risk in some women, while a low fat diet is associated with low estrogen levels (Hilakivi-Clarke and Clarke, 1998). However, other studies do not show an association between a high fat diet and breast cancer (Ames et al., 1995; Harris et al., 1997). In animals, lowered caloric intake inhibits mammary tumorigenesis (Lok, 1990; Gillette, 1997; Zhu, 1997; Rodriguez-Bufod, 1999; Zhu, 1999). Caloric restriction resulted in longer latency periods for palpable tumors, a decrease in final tumor incidence and a lower number of tumors. Lok (1990) showed that a 25% caloric restriction decreased cell proliferation in the mammary glands.

Recently, there has been much interest in the chemopreventive and anti-carcinogenic effect of phytoestrogens, a group of compounds with a chemical structure similar to estrogen. Nations that consume the largest amounts of phytoestrogens from soy foods, a rich source of phytoestrogens, have the lowest incidence and rate of death due to breast cancer (Barnes, 1998). A case-control study showed that a high intake of phytoestrogens could reduce the risk of breast cancer (Ingram et al., 1997). Genistein, a phytoestrogen found in soy products, has been extensively studied for its chemopreventive and antiproliferative activity. An early study showed that genistein can bind to sheep uterine and human breast cancer estrogen receptors \textit{in vitro} (Martin et al., 1978) and could interfere with the effects of estrogen. Another mechanism of action may be due to its ability to inhibit protein tyrosine kinase activity (Akiyama et al., 1987) and DNA-topoisomerase II (Markovits et al., 1989), both of which play an important role in cell proliferation. Cappelletini and colleagues (2000) showed that genistein can inhibit the growth of both hormone dependent and independent breast cancer cells by blocking the cell cycle at the G２M phase irrespective of the estrogen
receptor status. Balabhadrpathruni and colleagues (2000) also showed that genistein induced apoptosis in human breast cancer cells. Thus it has been demonstrated that genistein exerts its anti-carcinogenic effects by many different mechanisms.

Although the dietary factors that affect breast cancer risk have not been identified, good dietary habits can potentially go a long way in preventing breast cancer. Reducing consumption of red meat and total dietary fat while increasing consumption of fruits and vegetables may help reduce the risk of breast cancer.

2.3.2. Chemically Induced Tumorigenesis

Initiation, promotion and progression are the three steps involved in carcinogen-induced tumorigenesis (Figure 1-1) (Archer, 1992; Okey et al., 1998). Initiation results from the direct administration of carcinogen and is a rapid and irreversible process that causes damage to DNA. There are two types of carcinogen, ones that are direct acting and ones that require metabolic activation. Direct acting carcinogens are intrinsically electrophilic and can bind to nucleophilic sites in protein and nucleic acids. In contrast, some carcinogens require metabolism to become active. These carcinogens are called procarcinogens, which are converted to electrophilic metabolites by cytochrome P450 enzymes before they can bind to DNA. However, some of the metabolites are not carcinogenic and thus may be excreted without harm. When the direct acting carcinogen or the carcinogenic metabolites bind to DNA, it can cause DNA damage. The damaged DNA can be repaired by pre-replication excision repair, recombinational repair or post-replication repair. If the DNA is not repaired before it is replicated, the DNA lesions become permanent, forming initiated cells. These
initiated cells do not all become tumors since many will die through apoptosis. Cells that survive enter the next stage of tumorigenesis.

Promotion is the process where initiated cells, under the influence of tumor promoters such as hormones, are stimulated to divide to form pre-neoplastic cells and benign lesions (Archer, 1992; Okey et al., 1998). The promoters can cause an increase in cell division and a decrease in apoptosis, increasing the likelihood of tumorigenesis. This stage can occur long after initiation and can be reversed if the tumor promoters are removed.

The final stage of tumorigenesis is progression. In this stage the cancer cells become progressively more malignant and they acquire the ability to grow, invade local tissues and metastasize to distant locations.
Figure 1-1. General scheme describing the mechanism of chemical-induced tumorigenesis. Adapted from Archer (1992).
2.3.3. Rat Mammary Tumorigenesis as a Model for Humans

In rats, mammary tumors can be induced using carcinogens that act on the TEB and TD. When TEB and TD differentiate into AB, these can evolve into intraductal proliferation, carcinoma \textit{in situ} or invasive carcinoma. In humans, the TDLU can evolve into ductule carcinoma \textit{in situ} or invasive carcinoma. The difference between the pathogenic pathway in rats and humans is at the level of the TDLU. The TEB in rats are equivalent to the intralobular TD in humans. These areas are the most susceptible to neoplastic growth (Russo \textit{et al.}, 1990).

Carcinogen-induced rat mammary tumorigenesis in animals is widely used as a model for studying breast cancer. The two most widely used carcinogens to induce mammary tumorigenesis in rodents are 7,12-dimethylbenz(\(\alpha\))anthracene (DMBA) and N-methyl-N-nitrosourea (MNU) (Russo \textit{et al.}, 1990). DMBA and MNU are highly potent and with a single administration can cause a point mutation in the 61\textsuperscript{st} and 12\textsuperscript{th} codon respectively, in the \textit{ras} oncogene (Zarbl \textit{et al.}, 1985).

The primary target for DMBA is the rat mammary gland. DMBA is a pro-carcinogen that is activated by the cytochrome P450 located mainly in the endoplasmic reticulum of epithelial cells (Russo \textit{et al.}, 1983; Russo \textit{et al.}, 1982). The reactive electrophilic compounds interact preferentially with guanine and adenine, leading to excision repair and ultimately point mutations (Zarbl \textit{et al.}, 1985). This is the initiation event in tumorigenesis where an A\(\rightarrow\)T transversion at the second base of codon 61 occurs. The incidence of H-\textit{ras} mutation in codon 61 in DMBA induced mammary tumors is 23\% (Kito \textit{et al.}, 1996). DMBA induced mammary tumorigenesis is a good model for studying breast cancer for several reasons. Tumors can be induced in high incidence with short latency periods and little toxicity.
Tumor response is dose dependent and a single carcinogen dose is sufficient for tumor induction. As well, as in humans, tumors are hormone dependent. However, this model lacks local tumor invasion, distant metastasis and a high proportion of benign lesions (Shellabarger, 1972; Moon et al., 1976). As distant metastasis in humans usually leads to death, an animal model of breast cancer with tumors that have the ability to metastasize is desirable.

In contrast to DMBA, MNU induces mammary tumors that better simulate human breast cancer since it lacks the above deficiencies. Gullino et al. (1975) and McCormick et al. (1981) found that MNU-induced cancers metastasized to other organs such as the lung, spleen, liver and bone marrow. MNU is a direct-acting carcinogen that, once administered, is transformed into electrophilic alkylating agents at physiological pH (Archer, 1992). However, MNU is unstable at physiological pH because it is undetectable in rat blood 15 minutes after injection (Swann, 1968). In vitro studies have shown that MNU has a half-life of approximately eight minutes (Jensen et al., 1977). Upon exposure to MNU many different alkylation products are formed in DNA (Okey, 1998). The most common alkylation products, O\textsuperscript{6}-methylguanine and 7-methylguanine, are present in mammary glands and can be detected as soon as four hours after MNU injection (Cox and Irving, 1979). Both of these products are removed by O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT). The 7-methylguanine is removed at a faster rate than the O\textsuperscript{6}-methylguanine (Cox and Irving, 1979), resulting in a high frequency (≥99%) of point mutations in the second nucleotide of the H-ras codon 12, encoding glutamic acid instead of glycine (Zarbl et al., 1985).

Normally, \textit{ras} proteins transmit external signals such as growth factors from the plasma membrane to the nucleus. These proteins bind to GTP in order to become active.
They have an intrinsic GTPase activity to convert GTP to GDP so that the protein becomes inactive. However, a point mutation as described above results in the loss of the intrinsic GTPase activity causing the protein to be continually active and thus allowing it to stimulate growth in the absence of external stimuli (Engelbergs et al., 2000; Bos, 1989). Greater than 85% of rat mammary tumors induced by MNU harbor the H-ras mutation (Sukumar et al., 1983; Zarbl et al., 1985). Thus, this point mutation can be considered the initiation event in MNU induced mammary tumorigenesis. Kumar and colleagues (1990) showed that H-ras oncogenes were detected two weeks after carcinogen treatment and at least two months before the onset of the tumor. Induction of mammary carcinomas is primarily initiated in the epithelium of the TEB, which is the same as that observed in humans (Russo et al., 1990). This high susceptibility of TEB to neoplastic transformation is attributed to a high rate of cell proliferation, especially during puberty when the TEB are most actively differentiating to AB. Like breast cancer, MNU-induced mammary tumorigenesis is hormone dependent. Oophorectomy at the time of MNU treatment prevented the development of mammary tumors. In addition, oophorectomy performed when at least one tumor per rat was palpable resulted in regression of the tumor (Williams et al., 1981).

Both breast cancer in humans and mammary tumors in rats share many common immunocytochemical markers and histopathological characteristics (Russo et al., 1990). In addition, dietary fat intake, although it has remained controversial as a risk factor for breast cancer, has a great influence on mammary tumorigenesis in rats (Thompson et al., 1985; Klurfeld et al., 1987; Lok et al., 1990). Thus in humans and rats, genetics, hormones, exogenous agents and diet are risk factors for mammary tumorigenesis. These similarities in mammary gland development and mammary tumorigenesis between humans and rats make
the use of rats an adequate and an invaluable experimental model for understanding human breast cancer.

2.4. Zinc and Mammary Tumorigenesis

2.4.1. Plasma/serum zinc concentrations in humans

A possible link between zinc and mammary tumorigenesis is emerging. Plasma and serum zinc concentration has been evaluated in both animals and humans during mammary tumorigenesis. Plasma/serum zinc levels were lower in patients with breast cancer compared to healthy individuals (Chakravarty, Ghosh and Chowdhury, 1986; Gupta et al., 1991; Yucel et al., 1994; Sharma et al., 1994; Borella et al., 1997). The degree of hypozincemia was different depending on the stage of the cancer. Metastatic breast cancer patients had lower plasma/serum zinc levels than patients with cancer localized to the breast (Schlag et al., 1978; Holtkamp et al., 1990). Gupta and colleagues (1991) showed that serum zinc level in advanced breast cancer was lower than benign and early breast cancer.

However, plasma/serum zinc levels could not be used to distinguish breast cancer patients from controls (Piccinini et al., 1996; Dabek et al., 1994). Plasma/serum zinc levels in women with benign or malignant tumors were not statistically different from those in healthy controls (Garofalo et al., 1980; Koksoy et al., 1997; Magalova et al., 1999; Huang, Sheu, Lin 1999). Although Koksoy and colleagues (1997) did not find significance in the plasma zinc among the three groups of women in their study, they found that red blood cell zinc values were significantly increased in women with benign and malignant tumors compared to the controls.
In contrast to the above findings, Gupta and colleagues (1991) found serum zinc levels to be elevated in early breast cancer patients compared to controls. Cavallo and colleagues (1991) also found plasma/serum zinc levels to be higher in patients with primary cancer compared to healthy cancer free controls. They further compared the serum zinc levels of patients with different stages of breast cancer and found that there was no difference. Unlike the other studies, they assessed dietary zinc intake using questionnaires along with factors such as socioeconomic status, reproductive and medical history, all of which can affect zinc status.

The apparent inconsistency in these studies may be explained by the different factors that should be taken into consideration while evaluating plasma/serum zinc levels. It is well known that a wide range of nutritional and physiological factors affects plasma/serum zinc concentrations. Some researchers did not determine the menopausal status, age, stress level, and the stage of the cancer. The most important factor that was not determined in most of these studies was the diet of the patients in relation to the controls. Reduced zinc intake or an increased intake of foods that inhibit zinc absorption can affect the serum/plasma zinc levels (Sandström and Lönnerdal, 1989). The human body has a relatively constant level of plasma/serum zinc even when the diet varies. This is maintained by changes in the absorption and excretion efficiency of the gastrointestinal tract and by reductions of growth rate to conserve tissue zinc (Jackson, 1989). Plasma zinc levels are reduced when dietary zinc intake is so low that homeostatic processes are unable to establish normal zinc levels (Jackson, 1989).

One study that determined the relationship between diet in omnivores, vegetarians and breast cancer patients did not show a significant difference in the plasma zinc
concentration (Dabek et al., 1994). Interestingly there was no correlation between plasma zinc and zinc intake. However, they did show that pre-menopausal omnivores had a higher zinc intake compared to vegetarians and breast cancer patients. Therefore, it is important to consider dietary zinc intake.

2.4.2. Plasma/serum zinc concentrations in animals

Unlike in human studies, lowered plasma/serum zinc concentration in tumor bearing rats is clearer. Animal studies have shown that plasma zinc concentration is affected by tumor burden or level of dietary zinc intake. In transplanted animal mammary tumor models, tumor-bearing rats have a lower plasma zinc concentration compared to tumor free rats (Mills et al., 1981; Mills et al., 1984; Philcox et al., 1994). Tumor burden, which is the relative size of the mammary adenocarcinomas to the host weight in rats, also affects plasma zinc concentration. As tumor burden increased, the plasma zinc concentration decreased (Philcox et al., 1994). This effect was observed when tumor burden was less than 1% of body weight. These results suggest a relationship between zinc and tumor progression possibly because zinc is needed for growth, DNA, RNA and protein synthesis; as tumors grow the demand for zinc is increased.

Mills and colleagues (1981; 1984) showed that plasma zinc concentration in rats fed a zinc-deficient diet decreased 40-60% compared with that in rats fed the control diet. The dietary zinc concentration across these studies ranged from less than 1 mg/kg diet to less than 4 mg/kg diet for zinc deficient rats compared to 26 mg/kg diet to 35 mg/kg diet for controls. The decreased plasma zinc concentration was due to the lower levels of zinc provided in the diet.
However, when Philcox and colleagues (1994) fed the same diet to both control rats
and mammary adenocarcinoma-transplanted rats, plasma zinc was significantly lower in the
transplanted rats from as early as 19 hours post-injection. Although the amount of zinc
provided to both groups of rats was the same, the plasma zinc concentration was affected by
the tumor burden. They also reported that food intake was not significantly different. This
could be because the study was only seven days long and older rats were used as evidenced
by initial body weights between 180-200g.

2.4.3. Mammary gland and tumor zinc concentration

Mammary gland and tumor zinc concentrations have been investigated in both
animals and humans. In 1971, Mulay and colleagues reported a significantly higher zinc
level in breast cancer tissue than in normal breast tissue from the same individual. Other
studies that followed also showed similar results (Schwartz et al., 1974; Santoliquido et al.,
1976; Margalioth, Schenker and Chevion, 1983; Rizk and Sky-Peck, 1984; Ng et al., 1993;
Jin et al., 1999). The higher zinc concentration in malignant tissues may be because they
take up and retain more zinc than normal tissues. Tupper and colleagues (1955) performed a
study looking at the uptake and usage of zinc by normal and cancerous mammary gland
tissues in rats. They found that cancer tissues took up and retained more radioactive $^{65}$Zn
than normal tissues. Ng and colleagues (1993) speculate that a larger amount of zinc is
carried and deposited in cancerous tissue because of the increased blood supply relative to
normal tissue. Jin and colleagues (1999) reported that nuclear zinc concentration is higher
than plasma, membrane and cytoplasmic fractions.
2.4.4. Dietary zinc intake and mammary tumorigenesis

There are few studies on dietary zinc intake and mammary tumorigenesis in animals. However, early studies have used severe zinc deficiency in weaning male rats, which were implanted with lung cancer cells (McQuitty et al., 1970; DeWys et al., 1970). It was found that the survival rate of zinc deficient rats increased while tumor growth decreased. Mills and colleagues (1981) found that rats transplanted with Walker 256/M1 carcinomas on a zinc deficient diet of less than 1 mg/kg diet zinc resulted in a 70% decrease in tumor weights and increased tumor necrosis. When rats with established tumors were put on a diet consisting of less than 4 mg/kg diet zinc, tumor growth was decreased relative to tumor bearing pair fed rats on an adequate zinc diet. A limitation common to these early studies is the use of male rats, in which the effects of ovarian hormones on tumorigenesis is excluded. As well, established tumors were implanted and the effects of zinc on the earlier stages of tumorigenesis are unknown. Fischer and colleagues (1991), instead of using established tumors, used DMBA to induce mammary tumorigenesis in weaning female Sprague-Dawley rats. It was found that the number of mammary tumors was not significantly different in rats on adequate zinc diet (30 mg/kg diet) and high zinc diet (150 mg/kg diet). However, this study did not include lower zinc levels and other parameters of tumorigenesis were not reported.

Woo (1999) showed that marginally low zinc intake (3 mg/kg diet) exerted some suppressive effects on MNU-induced mammary tumorigenesis in rats. This inhibitory effect was partially due to reduced feed intake associated with zinc deficiency. However, in this study mammary tumorigenesis was induced in 21-day old rats before they had reached puberty. Furthermore, the rats began feeding on the assigned experimental diets on the same
day that mammary tumorigenesis was induced. It is well established that it takes several
weeks to develop zinc deficiency when rats are fed a marginally low zinc diet
(Panemangalore and Bebe, 1996). Thus the body zinc status in these rats was unlikely
reflecting their dietary zinc intake.

2.5. Pathology of Mammary Tumors

The classification of human and rat mammary tumors are similar. The staging,
grading and histology of tumors are performed in order to determine both the treatment and
prognosis for patients with breast cancer. The most common staging classification is the
TNM classification where T refers to tumor, N refers to nodes and M refers to the metastatic
status (Gillett, 1999). There are four stages, depending on the extent of tumor size, lymph
node involvement and metastasis. Stages 1 and 2 are usually treated surgically while Stages
3 and 4 are not usually treated surgically. The grading of tumors is assessed by examining
gland formation, pleomorphism (cell shape and size) and the number of mitosis. Grade 1, 2
and 3 have 85, 60 and 45% survival rates at 10 years from detection respectively (Stevens
and Lowe, 2000). The grading and staging of the tumor can also be used to determine if the
tumor is benign or malignant. Benign tumors grow faster than normal tissues but slower than
malignant tumors. These tumors grow in a regular tumor mass and are enclosed in a
connective tissue capsule. These tumors have better prognoses as they can be removed
surgically. However, malignant tumors grow faster, are very invasive, are rarely enclosed in
a capsule, and can spread to distant tissues and organs. These combine to give a poor
prognosis.
Histopathological analyses of NMU-induced rat mammary tumors have been reported by Thompson and colleagues (1992; 2000), who observed both benign and malignant tumors in MNU-induced mammary tumors. As well, the H-ras mutation was reported in malignant tumors while it was absent in benign tumors (Thompson et al., 2000). Malignant tumors are life threatening as they often lead to death by metastasizing to distant sites. Benign tumors do not metastasize and it is not life threatening. To date, the effects of zinc and the histopathology of mammary tumors have not been reported.

2.6. Summary

Both animal and human studies show a possible role of zinc in mammary tumorigenesis. Some studies have shown that zinc concentration is significantly higher in cancerous tissues than in non-cancerous tissue. Mammary tumorigenesis is characterized by the accumulation of gene mutations that eventually lead to uncontrolled cell growth. Since zinc is essential for over 300 metalloenzymes and some of which are important in cell proliferation, an altered dietary zinc intake could have an effect on mammary tumorigenesis. However, the effects of dietary zinc intake on mammary tumorigenesis are much understudied.
3. HYPOTHESIS AND RATIONALE

The hypothesis of my thesis research project is that dietary zinc levels will have an effect on mammary tumorigenesis in rats treated with chemical carcinogen MNU. MNU causes mutations in the H-ras oncogene and if cells harboring this mutation are not repaired or destroyed, cells harboring the mutation will proliferate to form initiated cells. Under the appropriate conditions, these initiated cells could eventually develop into tumors. Zinc is critical to several key events involved in growth, including DNA synthesis and cell proliferation. Zinc depletion in animals suppresses DNA synthesis (Williams and Chesters, 1970) while zinc repletion support DNA synthesis (Chesters et al., 1989). Since zinc is not stored in the body at an appreciate amount, dietary zinc intake has a great impact on body zinc status. It is thus possible that reduced dietary zinc intake, through its influence on body zinc status, could suppress cell proliferation and ultimately retard chemically induced mammary tumorigenesis in rats. In contrast, excessive dietary zinc intake could sustain cell proliferation and eventually promote chemically induced mammary tumorigenesis in rats.
4. OBJECTIVES

The overall objective of my thesis research project was to determine the effects of dietary zinc intake on the induction of mammary tumorigenesis by MNU in sexually mature female Sprague-Dawley rats.

The specific aims were:

1. To determine the effects of dietary zinc intake on MNU-induced mammary tumorigenesis in sexually mature rats.

2. To evaluate the effects of dietary zinc intake on the histological grade of MNU-induced mammary tumors in sexually mature rats.
5. REFERENCES


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CHAPTER 2
REDUCED ZINC INTAKE SUPPRESSED N-METHYL-N-NITROSOUREA-INDEuced MAMMARY TUMORIGENESIS IN SPRAGUE-DAWLEY RATS

(A revised version of this chapter is in preparation to be suited to Nutrition and Cancer)

1. INTRODUCTION

Zinc is vitally important to cell proliferation and growth. Zinc deprivation inhibits DNA synthesis and cell proliferation in cultured cells (Chesters et al., 1989; Prasad et al., 1996; MacDonald et al., 1998; Paski and Xu 2001, 2002) and in rats (James et al., 1987; Lawson et al., 1988). Tumorigenesis is characterized by dysregulation of cell proliferation. Since zinc is essential to cell proliferation, increased cell proliferation during tumorigenesis represents needs for a continuous and sufficient supply of zinc. Indeed, interrupted zinc supply during periods of low dietary zinc intake suppresses the growth of Walker 256/M1 carcinosarcoma in rats (McQuitty et al., 1970) and Lewis lung carcinoma in mice, and increases the survival rate of mice with leukemia (DeWys and Pories 1972). In contrast, increased zinc supply by switching from low zinc diet to zinc sufficient diet stimulates the growth of Walker 256/M1 carcinosarcoma in rats (Mills et al., 1981). Evidently, zinc is required to support tumor growth.

A possible link between zinc and mammary tumorigenesis is emerging. Plasma/serum zinc levels were lower in patients with breast cancer compared to healthy individuals (Chakravarty, Ghosh and Chowdhury, 1986; Gupta et al., 1991; Yucel et al., 1994; Sharma et al., 1994; Borella et al., 1997). The degree of hypozincemia appears to be related to the cancer stage. Plasma and serum zinc concentrations are lower in metastatic breast cancer patients than patients with cancer localized to the breast (Schlag et al., 1978;
Holtkamp et al., 1990). In addition, plasma and serum zinc concentrations are lower in patients with advanced breast cancer than in patients with benign and early breast cancer (Gupta et al., 1991). Similarly, in transplanted animal mammary tumor models, plasma zinc concentration is lower in tumor-bearing rats than in tumor free rats (Mills et al., 1981; Mills et al., 1984; Philcox et al., 1994). As the tumor burden increases (e.g. from 4.3% to 16.3%) the degree of hypozincemia becomes more severe (Philcox et al., 1994). More recently, we have shown accumulation of zinc in N-methyl-N-nitrosourea (MNU) -induced rat mammary tumors (Lee et al., 2003). This zinc accumulation appears to be due to decreased expression of ZnT-1, a zinc exporter, and increased expression of metallothionein, the main cellular zinc storage protein, suggesting that zinc homeostasis may be altered in MNU-induced rat mammary tumorigenesis to ensure an adequate supply of zinc to support tumor growth.

Information on the relationship between dietary zinc intake and mammary tumorigenesis is scarce. Mills et al. (1984) reported that low zinc intake inhibited mammary tumor growth in male rats implanted with mammary adenocarcinoma. However, mammary tumorigenesis is estrogen dependent (Welsch, 1985; Nandi et al., 1995). The implantation of mammary adenocarcinoma into male rats excludes the effects of ovarian hormones on the growth of mammary tumors. Moreover, implanted mammary tumors provide a model for studying the growth of established mammary tumors, but do not permit study of the influence of dietary zinc intake on the development of mammary tumors.

In our lab, we have previously showed that marginally low zinc intake (3 mg/kg diet) exerts some suppressive effects on MNU-induced mammary tumorigenesis in 21-day old rats (Woo and Xu, 2001). This inhibitory effect of marginally low zinc intake was partially due to reduced feed intake associated with zinc deficiency. MNU-induced mammary
tumorigenesis is also affected by hormones such as estrogen and progesterone (Welsch 1985; Nandi et al., 1995). Induction of mammary tumorigenesis in 21-day old rats was before they had reached puberty. Furthermore, the rats began feeding on the low zinc diet on the same day that mammary tumorigenesis was induced. It is well established that it takes several weeks to reach a zinc deficient body status when rats are fed a marginally low zinc diet (Panemangalore and Bebe, 1996). Thus, these rats were unlikely zinc deficient at the time mammary tumorigenesis was induced. Therefore, the effects of dietary zinc intake on mammary tumorigenesis require further investigation.

The objective of this study was to determine the effects of dietary zinc intake on MNU-induced mammary tumorigenesis in sexually mature female rats. In this study, MNU-induced mammary tumorigenesis was assessed using tumor incidence, tumor number, tumor size (both tumor weight and volume), tumor burden, tumor multiplicity, and tumor latency as well as histological evaluation. Our observations showed that a marginally low intake of zinc suppressed MNU-induced mammary tumorigenesis as indicated by a lowered tumor incidence, tumor number and tumor multiplicity.

2. MATERIALS AND METHODS

2.1 Dietary Treatments and Animals

Twenty one-day old female Sprague-Dawley rats (The University of British Columbia Animal Care Centre) with an average body weight of 49 g were assigned to one of the following dietary treatment groups: low zinc (3 mg zinc/kg diet; Z3), adequate zinc (12 mg zinc/kg diet; Z12) or high zinc (155 mg zinc/kg diet; Z155) groups. The adequate zinc diet was to meet the recommended dietary zinc intake for rats (National Research Council,
1995). The low zinc diet was designed to produce marginally low zinc intake (Panemangalore and Bebe, 1996; Woo and Xu, 2001). The high zinc diet was aimed to produce high zinc status (Panemangalore and Bebe, 1996). The diets were formulated and prepared as previously reported (Woo and Xu, 2001). Rats in the Z3, Z12 and Z155 groups were fed with their respective diet *ad libitum*. Marginally low zinc intake suppressed feed intake (Woo and Xu, 2001). To overcome this potential confounding factor, two pair-fed groups, pair-fed to adequate zinc (PZ12) and pair-fed to high zinc (PZ155) groups, were included. Rats in PZ12 and PZ155 were given the adequate and high zinc diet, respectively, at the same amount consumed by the rats in the Z3 group during the previous 24 hours. On day one of the experiment, rats in the PZ12 and PZ155 groups were given 5 g of their assigned diet. The rats were maintained on their assigned diet for a total of 18 weeks with 25 rats per group, except the Z3 group where there were 33 rats. The higher number of rats in the Z3 group was designed to ensure adequate replications at the end of the feeding trial. The rats were individually housed in stainless steel cages in a temperature and humidity regulated room with a 12 hour light/dark cycle with free access to double deionized water. All animals were cared for in compliance with the Canadian Council of Animal Care’s Guide.

### 2.2 Induction of Mammary Tumorigenesis

All rats were fed on their assigned diets from 21-days to 50-days of age at which time the rats were injected intraperitoneally with MNU (Sigma, Oakville, Ontario) at a dose of 50 mg/kg body weight. MNU was prepared and administered as described earlier (Thompson and Adlakha, 1991). Briefly, MNU was dissolved in cold saline (4°C, 0.9% NaCl) and 0.05% acetic acid with a pH of 4 to increase its stability. Upon preparation, the MNU
solution was kept on ice and protected from light during the injection period to prevent MNU from breaking down. All MNU solution was used within 30 min after its preparation. We chose the 50 days of age for MNU injection for two reasons. First, rats reach sexual maturity between the ages of 45 and 60 days (Russo, et al., 1990). During puberty, the mammary epithelium is proliferating most rapidly, which ensures a significant induction of mammary tumors. Second, it usually requires about three to four weeks for rats to achieve the desired zinc status to reflect their corresponding dietary zinc intake (Panemangalore and Bebe, 1996). This is especially critical to the Z3 group.

2.3 Assessment of Zinc Status

Body zinc status was assessed by host body weight gain, feed intake, and plasma zinc concentration. Body weight was monitored throughout the feeding trial to assess the growth. Host body weight gain was calculated using the following formula: host body weight gain = (final body weight - initial body weight) - tumor weight. Feed intake of the rats in Z12 and Z155 groups were monitored weekly. Feed intake of the rats in the Z3 group was monitored daily to determine the amount of feed fed to the rats in PZ12 and PZ155 groups. At the end of the feeding trial, plasma was prepared (2,000 rpm, 20 min, 4°C) and stored at −80°C until analysis. The plasma samples were diluted with 0.1 N nitric acid to an appropriate concentration before the determination of zinc concentration using flame atomic absorption spectroscopy (Perkin Elmer, Model 2380, Norwalk, CT).
2.4 Assessment of Mammary Tumorigenesis

Beginning at six weeks post-MNU injection, all rats were palpated weekly to monitor the development of mammary tumors. After 14 weeks post-MNU injection, the rats were anesthetized and all mammary tumors were extracted. The tumor locations were confirmed by checking against the palpation records. Tumors detected prior to necropsy were counted as palpable tumors. Some tumors were very small and discovered at necropsy and were counted as non-palpable tumors. Total tumor was calculated as the sum of palpable and non-palpable tumors. Each tumor was individually weighed and the dimensions were recorded during necropsy. When the tumor size was sufficiently large, a segment of the tumor was frozen in liquid nitrogen for biochemical analyses, while the remaining part was fixed in 10% buffered formalin for histological analyses.

Mammary tumorigenesis was assessed by determining tumor incidence, tumor numbers, tumor size, tumor burden, tumor multiplicity, and tumor latency. The tumor incidence was the percentage of rats that developed mammary tumors. Tumor incidence and tumor numbers were calculated using both palpable and total mammary tumors. Tumor size was assessed by both tumor weight and tumor volume \((w \times h \times l \times \pi/6)\). Tumor burden was calculated as the percentage of total mammary tumor weight in a tumor-bearing rat relative to the final host body weight, which was the final body weight less the total tumor weight. Tumor multiplicity was calculated as the average number of palpable or total mammary tumors per tumor-bearing rat. Tumor latency period was the average time that the first mammary tumor was palpated in a tumor-bearing rat in each dietary treatment group.
2.5 Classification of Mammary Tumors

For histological evaluation, tumors fixed in buffered formalin were randomly selected based on the following principles to ensure adequate representation. If the tumor-bearing rat had ≤ 2 tumors, all tumors were used. If the tumor-bearing rat had ≥ 2 palpable tumors and no non-palpable tumors, or had ≥ 2 non-palpable tumors and no palpable tumors, 2 tumors were randomly selected. If the rat had both palpable and non-palpable tumors, 1 palpable and 1 non-palpable tumor were randomly selected. The classification of tumors was performed independently by two individuals, both of whom were blinded.

Hematoxylin and eosin (H&E) stained tumor sections (5 μm) were first classified as benign or malignant tumors using the established diagnosis (Table 2-1; Nowak and Handford, 1994). Malignant tumors were then classified according to their architectural structure as described by Young and Hallowes (1973) as well as Russo et al. (1989). Briefly, papillary carcinomas have fibrovascular cores from which the epithelial cell masses grow forming papillary projections into and almost filling the lumen. Depending on the thickness and the cytological characteristics of the epithelial cell mass, papillary carcinomas are further classified as grade I or grade II (Table 2-2; Russo et al., 1989). Ductal carcinomas are proliferations of malignant epithelial cells confined to mammary ducts. Comedo carcinomas are distended ductal structures lined by neoplastic epithelium surrounding necrotic debris. Cribiform carcinomas are solid sheets of neoplastic epithelium with variable size and shape of secondary lumina. Comedo and cribiform carcinomas are usually associated with other patterns.

Tumor classification based on the architecture is sometimes difficult because multiple patterns are often present in the same tumor. Thus, we further classified the malignant
tumors into high-grade or low-grade malignant tumors. Tumors that were many cell layers thick, had prominent mitosis, and marked pleomorphism were classified as high-grade tumors. Low-grade tumors usually have three or fewer cell layers, low mitosis and moderate pleomorphism.

In addition, the invasiveness of the mammary tumors was also studied using the following criteria. Invasive tumors are characterized by infiltration to the surrounding structures (e.g. stroma, muscle, dermis, fat and nerves), and presence of fibrosis, tissue scarring resulting from nonspecific tissue repair due to chronic inflammation, broken basement membrane, inflammation, and desmoplastic reaction in the stroma surrounding the malignant tumor. In contrast, in situ mammary tumors are classified based on the absence of invasion to the surrounding structures and fibrosis, and presence of intact basement membrane, and less inflammation and desmoplastic reaction in the stroma. The histological images were captured using a digital camera (JVC, Model KY-F70).

2.6 Statistical Analysis

Tumor incidence was analyzed using the Chi-square test ($p<0.05$). The differences between dietary treatment means for other parameters were analyzed using one-way analysis of variance followed by the least significant difference test ($p<0.05$; The SAS System for Windows Release 6.12).
3. RESULTS

3.1 Body zinc status

Body zinc status was assessed by feed intake, host body weight gain, and plasma zinc concentration (Table 2-3). Feed intake in Z3 rats was reduced by 17 and 27% compared with Z12 and Z155 rats, respectively ($p<0.05$). Host body weight gain in Z3 rats was also lowered by 10 and 22% compared with Z12 and Z155 rats, respectively ($p<0.05$). However, at the same level of feed intake, Z3 rats gained a similar amount of host body weight as its pair-fed controls (Z3 rats vs. PZ12 and PZ155 rats). Moreover, although they consumed the same diet, host body weight gain in the pair-fed control rats was reduced 6 and 20% in PZ12 and PZ155 rats, respectively, compared with their corresponding *ad libitum* control rats ($p<0.05$). These data demonstrated that a reduced host body weight gain in Z3 rats compared with Z12 and Z155 was due to reduced feed intake associated with low zinc intake rather than reduced dietary zinc intake per se. Similarly, the feed efficiency (feed intake : host body weight gain ratio) was also affected by feed intake rather than dietary zinc level. Plasma zinc concentration in Z3 rats was about 50% of that in both pair-fed and *ad libitum* controls ($p<0.05$), but was not significantly different between the pair-fed control rats and their corresponding *ad libitum* control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats).

Compared with Z12 rats, Z155 rats consumed more feed and gained more body weight along with displaying a lower feed efficiency ($p<0.05$). However, at the same level of feed intake, the difference in dietary zinc level had no effect on host body weight gain and feed intake to body weight gain ratio (PZ12 rats vs. PZ155 rats). Moreover, plasma zinc concentration was the same between Z12 and Z155 rats. These data indicated that a higher
host body weight gain in Z155 rats resulted from higher feed intake rather than a higher dietary zinc level.

3.2 Influence of dietary zinc intake on the incidence of mammary tumors

The incidence of mammary tumors includes the overall incidence of both palpable and total tumors (Table 2-4). The overall incidence of palpable tumors in PZ12, Z12, PZ155 and Z155 rats was about 2.5 times (2.3 – 2.5 times) of that in Z3 rats (p<0.05). The overall incidence of total tumors, which included both palpable and non-palpable tumors, in PZ12, Z12, PZ155 and Z155 rats was about 2 times (1.7 – 1.9 times) of that in Z3 rats (p<0.05). The overall incidence of both palpable tumors and total tumors was not significantly different between the pair-fed control rats (PZ12 rats vs. PZ155 rats), between the pair-fed control rats and their corresponding ad libitum control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats), and between the ad libitum control rats (Z12 rats vs. Z155 rats).

The temporal pattern of appearance of palpable tumors is shown in Figure 2-1. Cumulative incidence of palpable tumors in Z3 rats was significantly lower than that in Z12 and Z155 rats starting from week eight post-MNU-injection to the end of the feeding trial (p<0.05). When compared with the pair-fed control rats, cumulative incidence of palpable tumors in Z3 rats was not significantly different from that in PZ12 and PZ155 rats until week eleven and ten post MNU-injection, respectively (p<0.05). Cumulative incidence of palpable tumors in the pair-fed control rats was significantly lower than that in their corresponding ad libitum control rats from week eight to eleven post-MNU-injection (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats; p<0.05), but was not significantly different thereafter. Cumulative incidence of palpable tumors was not significantly different between Z12 and Z155 rats.
3.3 Influence of dietary zinc intake on the number of mammary tumors

The number of mammary tumors includes the overall number of both palpable tumors and total tumors (Table 2-4). The overall number of palpable tumors in PZ12, Z12, PZ155, and Z155 rats was 2.4, 4.6, 2.8, and 4.8 times of that in Z3 rats, respectively. The overall number of palpable tumors in PZ12 and PZ155 rats was reduced by 48 and 42%, respectively, compared with their corresponding ad libitum control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats), but was very similar between the pair-fed control rats (PZ12 rats vs. PZ155 rats), and between the ad libitum control rats (Z12 rats vs. Z155 rats). The temporal pattern of cumulative palpable tumor number was very similar to the overall number of palpable tumors with a lower number of palpable tumors in Z3 rats than that in both the pair-fed (Z3 rats vs. PZ12 and PZ155 rats) and ad libitum control rats (Z3 rats vs. Z12 and Z155 rats), and that in the pair-fed control rats than in their corresponding ad libitum control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats; Figure 2-2).

Similarly, the overall number of total tumors in PZ12, Z12, PZ155, and Z155 rats was 2.4, 4.4, 2.8, and 4.2 times of that in the Z3 rats, respectively. The overall number of total tumors in PZ12 and PZ155 rats was reduced by 46 and 34%, respectively, compared with their corresponding ad libitum control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats), but was very similar between the pair-fed control rats (PZ12 rats vs. PZ155 rats), and between the ad libitum control rats (Z12 rats vs. Z155 rats).
3.4 Influence of dietary zinc intake on tumor multiplicity, burden, and latency

The multiplicity of both palpable and total tumors in Z12 and Z155 rats was about 3 times (2.6 – 3.3 times) of that in the Z3 rats, and was about 2 times (1.6 – 2.1 times) of that in their corresponding pair-fed control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats; p<0.05; Table 2-4). The multiplicity of palpable tumors in Z3 was similar to the pair-fed control rats (Z3 rats vs. PZ12 and PZ155 rats). However, the multiplicity of total tumors in PZ12 and PZ155 rats were 1.7 and 2.1 times, respectively, of that in the Z3 rats (p<0.05). The multiplicity of both palpable and total tumors was different between the pair-fed control rats (PZ12 rats vs. PZ155 rats), and between the ad libitum control rats (Z12 rats vs. Z155 rats).

Tumor burden in Z12 and Z155 rats was 11.9 and 6.2 times, respectively, of that in Z3 rats (p<0.05; Table 2-4). Tumor burden was significantly lower in PZ155 rats than that in Z155 rats (p<0.05), but was similar between Z3 rats and the pair-fed control rats (Z3 rats vs. PZ12 and PZ155 rats), between PZ12 and Z12 rats, and between the ad libitum control rats (Z12 rats vs. Z155 rats).

Tumor latency was significantly longer in Z3 rats than that in Z12 and Z155 rats (p<0.05; Table 4), and longer in the pair-fed control rats than that in their corresponding ad libitum control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats; p<0.05). However, tumor latency was similar between the pair-fed control rats (PZ12 rats vs. PZ155 rats) and between the ad libitum control rats (Z12 rats vs. Z155 rats).
3.5 Influence of dietary zinc intake on tumor size

The tumor size was assessed by both tumor volume and tumor weight (Table 2-5). The mean tumor volume in Z12 and Z155 rats was 3.6 and 2.6 times, respectively, of that in the Z3 rats \((p<0.05)\). The mean tumor volume was significantly higher in Z155 rats than that in PZ155 rats \((p<0.05)\), but was similar between Z3 rats and the pair-fed control rats (Z3 rats vs. PZ12 and PZ155 rats), between PZ12 rats and Z12 rats, and between the \textit{ad libitum} control rats (Z12 rats vs. Z155 rats). The median tumor volume in PZ12, Z12, PZ155 and Z155 rats was 1.7, 2.3, 1.4, and 2.8 times, respectively, of that in the Z3 rats.

Similarly, the mean tumor weight in Z12 and Z155 rats was 3.5 and 2.3 times of that in the Z3 rats \((p<0.05; \text{Table 2-5})\). The mean tumor weight was significantly heavier in Z155 rats than that in PZ155 rats \((p<0.05)\), but was similar between Z3 rats and the pair-fed control rats (Z3 rats vs. PZ12 and PZ155 rats), between PZ12 and Z12, and between the \textit{ad libitum} control rats (Z12 vs. Z155 rats). The median tumor weight in PZ12, Z12, and Z155 rats was three times of that in Z3 rats, but was the same between Z3 and PZ155 rats, between the pair-fed control rats and their corresponding \textit{ad libitum} control rats (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats), and between the \textit{ad libitum} control rats (Z12 rats vs. Z155 rats).

3.6 Effects of dietary zinc intake on mammary tumor grade

Mammary tumors were classified for benign or malignant and tumor grade, using the criteria outlined in Tables 2-1 and 2-2, as well as tumor invasiveness. Administration of MNU resulted only in malignant tumors (Figure 2-3) and no benign tumors. Mixed areas of benign and malignant were present in two tumors examined: one was Grade 1 papillary and
the other was Grade 2 papillary (Figure 2-4). These two tumors were scored as malignant because of the presence of malignant area. The structure of the tumors in different dietary treatment groups is shown in Table 2-6. A majority of the tumors were Grade 1 (14%) and Grade 2 (49%) papillary or mixed papillary carcinomas (29%). The remaining carcinomas made up 8% of the total tumors analyzed.

The effect of dietary zinc intake on the grade of the tumors is shown in Table 2-7. There were no dietary effects on the tumor grade. The grade of the tumors was not affected by the time at which the tumor was detected, but it was affected by the location of the tumor. Most of the low-grade tumors (81%) were located in the abdominal-inguinal area, while the rest (19%) were located in the cervical-thoracic area. In contrast, most of high-grade tumors (57%) were located in the abdominal-inguinal area, while the rest (43%) were located in the cervical-thoracic area.

The invasiveness of the tumor was also determined for a majority of the tumors (71%), but for 29% of the tumors invasiveness could not be determined because these tumors did not have surrounding tissues to determine invasion. Therefore, they were excluded in the analysis with invasiveness. The invasiveness of the tumors was not affected by the dietary zinc treatment, but was affected by the location of the tumors. The \textit{in situ} tumors in the abdominal-inguinal and cervical-thoracic areas were 59 and 41%, respectively. The invasive tumors in the abdominal-inguinal and cervical-thoracic areas were 50 and 50%, respectively. The grade was affected by the invasiveness of the tumor. In low grade tumors, \textit{in situ} tumors and invasive tumors were 79 and 21%, respectively. In contrast, in high-grade tumors, \textit{in situ} tumors and invasive tumors were 26 and 74%, respectively. Since the abdominal-inguinal area had a higher percentage of low-grade tumors compared to the
cervical-thoracic area, it follows that the abdominal-inguinal area also had a higher percentage of in situ tumors. This was confirmed by having a higher percentage of in situ tumors in the low grade.

4. DISCUSSION

4.1 Development of marginal zinc deficiency

In this study, consumption of the low zinc diet significantly reduced feed intake, host body weight gain, and plasma zinc concentration in the Z3 rats compared with the adequate zinc control rats (Z12 rats). These results clearly demonstrated zinc deficiency status in Z3 rats. However, when compared with PZ12 rats, Z3 rats gained the similar amount of host body weight while plasma zinc concentration was significantly lower. The PZ12 rats consumed the same zinc adequate diet as the Z12 rats, but at the same amount that Z3 rats consumed. Therefore, reduced host body weight gain in the Z3 rats compared to the Z12 rats was caused by reduced feed intake associated with zinc deficiency, rather than zinc deficiency per se. Appearance of zinc deficiency signs is a function of dietary zinc intake. In severe zinc deficiency (i.e. feeding diets containing < 1 mg zinc/kg diet), alopecia, feed refusal, growth retardation and low plasma/serum zinc concentration are common signs of zinc deficiency in rats (Hammermueller et al., 1987; Taylor et al., 1988; Xu and Bray, 1994; Xu et al., 1994a, 1994b). In contrast, mild or marginal zinc deficiency (i.e. feeding diets containing 3 mg zinc/kg diet) results in reduced feed intake and low plasma zinc concentration, while body weight gain is not affected (Woo and Xu, 2001). Moreover, dietary zinc intake at 1.5 – 2 mg is known to develop marginal zinc deficiency in rats (Panemangalore and Bebe, 1996). Collectively, reduced feed intake and lower plasma zinc
concentration along with a similar host body weight gain confirmed the marginal zinc deficiency status in the Z3 rats.

4.2 Low dietary zinc intake suppressed MNU-induced mammary tumorigenesis

Cumulative and overall tumor incidences, cumulative and overall tumor numbers, and multiplicity of total tumors were significantly lower in the Z3 rats than that in the adequate zinc control rats (PZ12 and Z12 rats). Moreover, since the dietary zinc content in the low zinc and adequate zinc diets was 3 and 12 mg zinc/kg diet, and both the Z3 and PZ12 rats consumed the same amount of feed, dietary zinc intake in the PZ12 rats was 4 times that in the Z3 rats while the intake of all other nutrients and energy were the same between these two groups of rats. This inhibitory effect of low zinc intake on MNU-induced mammary tumorigenesis is supported by earlier studies. Mills and co-workers (1984) reported that low zinc intake (< 4 mg zinc/kg diet) results in a 35% inhibition of mammary tumor growth in male rats transplanted with mammary adenocarcinoma when compared to the pair-fed control rats fed an adequate zinc diet (30-50 mg zinc/kg diet). More recently, Woo and Xu (2001) have shown that low dietary zinc intake (3 mg zinc/kg diet) significantly reduced the incidence of palpable tumors and total number of tumors by 78 and 53%, respectively when compared to pair-fed-adequate-zinc-intake rats (12 mg zinc/kg diet). Therefore, reduced tumor incidence, tumor numbers and tumor multiplicity collectively demonstrated that low dietary zinc intake suppressed MNU-induced mammary tumorigenesis in rats. However, the mechanisms involved are not known.

MNU is an alkylating agent. The carcinogenicity of MNU is due to its ability to induce a $G \rightarrow A$ mutation in the second codon of the H-ras oncogene (Sukumar et al., 1983).
Once the mutation occurs, the initiated cells can undergo cell death, DNA repair to form normal cells, or rapid cell proliferation provided that there is sufficient supply of nutrients required for growth. Either increased death or decreased proliferation of the initiated cells, or a combination of these two events can result in a lower tumor incidence. Zinc is critical to both cell death and cell proliferation.

Cell death can occur through either necrosis or apoptosis. Zinc is important to both. For example, in human breast cancer MDA-MB-231 cells, depression of intracellular zinc by using N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), an intracellular zinc chelator, resulted in a 15% increase in necrosis (Wu, 2003). Zinc is considered as a physiological regulator of apoptosis. In rats, zinc deficiency increases apoptosis in the crypt region of the small intestine (Elmes, 1977) and a variety of cells (Nodera et al., 2001). In vitro, culturing lymphoid and myeloid cells in low zinc media increased apoptosis (Martin et al., 1991). Similarly, zinc deprivation by using EDTA (Sakabe et al., 1998) and TPEN (Zalewski et al., 1993) induces apoptosis in mouse neuroblastoma cells and thymocytes, respectively. In human breast cancer MDA-MB-231 cells, TPEN-induced zinc depletion also increases apoptosis (Wu, 2003). In contrast, zinc supplementation suppresses apoptosis in thymocytes (Sellins and Cohen, 1987) and fibroblasts, macrophage, T lymphocytes, and leukaemic cells (Waring et al., 1990; Barbieri et al., 1992; McGowan et al., 1994). Clearly, zinc deprivation induces apoptosis while zinc supplementation suppresses apoptosis.

Zinc is an essential nutrient required for DNA synthesis, cell proliferation, and growth. In cultured cells, zinc deprivation inhibits DNA synthesis and cell proliferation (Chester et al., 1989; Prasad et al., 1996; MacDonald et al., 1998; Paski and Xu 2001, 2002). For example, zinc depletion using diethylenetriaminopenta acetate (DTPA), an
extracellular divalent cation chelator, causes a nearly complete suppression of DNA synthesis in 3T3 cells (MacDonald et al., 1998). This suppressive effect of DTPA on DNA synthesis can be reversed by adding zinc, but not other metals such as calcium or iron, to the culture media, which indicates that this DTPA-induced suppression of DNA synthesis is zinc specific. Besides chelation, zinc deprivation using nutritional means also results in reduced DNA synthesis and cell proliferation in 3T3 cells (Paski and Xu, 2001). Zinc repletion increases DNA synthesis and cell proliferation in 3T3 cells. Lee et al. (2003) recently have shown that zinc concentration in MNU-induced mammary tumors in rats is higher than that in mammary glands through an increased expression and presence of metallothionein, the main zinc storage protein, coupled with a reduced expression of ZnT-1, an zinc exporter. This altered expression of metallothionein and ZnT-1 suggests that zinc homeostasis might be altered in MNU-induced mammary tumors in rats. Since adequate zinc supply is essential to cell proliferation and tumors are characterized by uncontrolled cell proliferation, zinc accumulation in MNU-induced mammary tumors likely is part of the strategies to ensure an adequate zinc supply to sustain tumorigenesis. Thus, we speculate that zinc deficiency mediated increase in apoptosis and reduced cell proliferation might collectively play a role in the inhibitory effect of low dietary zinc intake on MNU-induced mammary tumorigenesis in rats. Further studies are required to test these hypotheses.

4.3 Inhibitory effects of reduced feed intake on MNU-induced mammary tumorigenesis

Incidence of palpable tumors in the pair-fed control rats (PZ12 and PZ155 rats) was significantly lower than that in their corresponding ad libitum control rats (Z12 and Z155 rats) until week eleven post-MNU injection. From week twelve post-MNU injection to the
end of the feeding trial, the incidence of palpable tumors was the same between the pair-fed control rats and their corresponding *ad libitum* control rats. This correlated well with the feed intake. Feed intake in the pair-fed control rats was reduced by an average of 25% during the first eleven weeks post-MNU injection and of 11% from week twelve post-MNU injection to the end of the feeding trial. These results were similar to that of ThyagaRajan and colleagues (1993). They showed that the tumor burden and tumor multiplicity is significantly lower in rats with 50% of feed restriction two months after DMBA administration.

Tumor number, multiplicity of palpable tumors, tumor latency, tumor size (both tumor volume and tumor weight), and tumor burden were lower in the Z3 rats than that in the Z12 rats. However, these parameters were not significantly different between the Z3 and PZ12 rats. Since the Z3 and PZ12 rats consumed the same amount of feed, and the amount of feed these two groups of rats consumed was lower than the amount consumed by the Z12 rats, the lower tumor number, multiplicity of palpable tumors, tumor latency, tumor size (both tumor volume and tumor weight), and tumor burden observed in the Z3 rats were apparently caused by reduced feed intake associated with low zinc intake.

Moreover, tumor numbers, tumor multiplicity and tumor latency were lower in the pair-fed control rats than in their corresponding *ad libitum* control rats regardless of dietary zinc intake (PZ12 rats vs. Z12 rats; PZ155 rats vs. Z155 rats). In addition, tumor size, both tumor volume and tumor weight, and tumor burden were also lower in the PZ155 rats than that in the Z155 rats. The pair-fed control rats consumed the same diet as their corresponding *ad libitum* control rats at a lower amount. Thus, the differences in these parameters between the pair-fed control rats and the *ad libitum* control rats were also caused
by reduced feed intake associated low zinc intake. Clearly, these observations demonstrated that reduced feed intake associated with low zinc intake inhibited MNU-induced mammary tumorigenesis in rats.

4.4 Lack an effect of zinc supplementation on MNU-induced mammary tumorigenesis

When the high zinc intake rats were compared with the adequate zinc intake rats (PZ155 rats vs. PZ12 rats; Z155 rats vs. Z12 rats), all of the endpoints used to assess MNU-induced mammary tumorigenesis were the same, suggesting that zinc supplementation had no effect on the outcome of MNU-induced mammary tumorigenesis in rats. These observations are consistent with earlier reports. Fischer et al. (1991) also reported that feeding a high zinc diet (150 mg zinc/kg diet) has no effect on the 7,12-dimethylbenz(a)anthracene (DMBA) -induced mammary tumors in rats. Similarly, Woo (1999) also observed that zinc supplementation at 155 mg zinc/kg diet has no effect on the outcome of MNU-induced mammary tumorigenesis in rats. This lack of an effect of zinc supplementation on MNU-induced mammary tumorigenesis perhaps reflects the fact that, although zinc deficiency suppresses growth, there is no evidence showing zinc supplementation promoting growth.

In summary, low zinc intake reduced MNU-induced mammary tumorigenesis in sexually mature female rats. Some of the inhibitory effects of low zinc intake on MNU-induced mammary tumorigenesis were the result of reduced feed intake associated with low zinc intake, rather than low zinc intake per se. Zinc supplementation, however, had no effect on MNU-induced rat mammary tumorigenesis.
Table 2-1. Criteria for diagnosis of benign and malignant tumors\(^1\).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell structure</td>
<td>Well differentiated and uniform</td>
<td>Poorly differentiated, cellular and nuclear pleomorphism</td>
</tr>
<tr>
<td>Invasive growth</td>
<td>Uncommon</td>
<td>Typical</td>
</tr>
<tr>
<td>Capsule</td>
<td>Typical</td>
<td>Rare, incomplete if present</td>
</tr>
<tr>
<td>Mitosis</td>
<td>Few</td>
<td>Many</td>
</tr>
</tbody>
</table>

\(^1\)Adapted from Nowak and Handford 1994.
Table 2-2. Criteria for diagnosis of papillary carcinoma Grades I and II

<table>
<thead>
<tr>
<th>Components</th>
<th>Grade I</th>
<th>Grade II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrovascular core</td>
<td>Prominent</td>
<td>Sparse</td>
</tr>
<tr>
<td>Epithelium</td>
<td>3 layers thick</td>
<td>5-10 layers thick</td>
</tr>
<tr>
<td>Micropapillae</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Cytological characteristics</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td>Nucleolus mitoses</td>
<td>Inconspicuous, scarce</td>
<td>Prominent</td>
</tr>
</tbody>
</table>

1 Adapted from Russo et al., 1989.
**Table 2-3.** Effects of dietary Zn intake on body zinc status in rats injected with N-methyl-N-nitrosourea

<table>
<thead>
<tr>
<th></th>
<th>Dietary treatments</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z3</td>
<td>PZ12</td>
<td>Z12</td>
<td>PZ155</td>
<td>Z155</td>
</tr>
<tr>
<td>Feed intake (g)</td>
<td>1,469 ± 33&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1,414 ± 16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1,778 ± 33&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1,431 ± 17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2,003 ± 47</td>
</tr>
<tr>
<td>Host body weight gain (g)</td>
<td>223 ± 6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>220 ± 5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>243 ± 7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>230 ± 4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>286 ± 10</td>
</tr>
<tr>
<td>Feed intake: Host body weight gain</td>
<td>6.6 ± 0.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>6.6 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.8 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.3 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>Plasma zinc (μg/ml)</td>
<td>0.9 ± 0.1&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values represent the mean ± SEM (n = 25 rats, except Z3, PZ155 and Z155 where n = 33, 23, and 24 rats, respectively). Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed-with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).

<sup>a</sup> Significantly different from Z12 group (p < 0.05).

<sup>b</sup> Significantly different from Z155 group (p < 0.05).

<sup>c</sup> Significantly different from PZ12 and PZ155 groups (p < 0.05).

<sup>d</sup> Significantly different from their corresponding ad libitum control group (PZ12 vs. Z12; PZ155 vs. Z155; p < 0.05).

<sup>e</sup> Significantly different from Z155 group (p < 0.05).
Table 2-4. Effect of dietary zinc intake on the induction of mammary tumors by N-methyl-N-nitrosourea

<table>
<thead>
<tr>
<th></th>
<th>Dietary treatments</th>
<th>Z3</th>
<th>PZ12</th>
<th>Z12</th>
<th>PZ155</th>
<th>Z155</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall tumor incidence (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palpable tumor</td>
<td></td>
<td>33&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>76</td>
<td>80</td>
<td>83</td>
<td>79</td>
</tr>
<tr>
<td>Total tumor&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>46&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>80</td>
<td>84</td>
<td>87</td>
<td>79</td>
</tr>
<tr>
<td>Overall tumor number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palpable tumor</td>
<td></td>
<td>20</td>
<td>47</td>
<td>91</td>
<td>55</td>
<td>95</td>
</tr>
<tr>
<td>Total tumor</td>
<td></td>
<td>28</td>
<td>66</td>
<td>123</td>
<td>77</td>
<td>117</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palpable tumors</td>
<td></td>
<td>1.8 ± 0.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.4 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.6 ± 0.8</td>
<td>2.7 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>Total tumor</td>
<td></td>
<td>1.9 ± 0.3&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>3.3 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.9 ± 0.9</td>
<td>3.9 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Tumor burden (%)</td>
<td></td>
<td>0.3 ± 0.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.2 ± 0.7</td>
<td>3.8 ± 1.3</td>
<td>1.1 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>Tumor latency (weeks)</td>
<td></td>
<td>12.8 ± 0.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>11.4 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.3 ± 0.6</td>
<td>11.8 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.4 ± 0.4</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM. Twenty-five rats were assigned to each dietary treatment group, except Z3, PZ155 and Z155, where there were 33, 23, and 24 rats, respectively, in each group. The overall tumor incidence was the percentage of rats that developed mammary tumors. Tumor multiplicity was the average number of tumors per tumor-bearing rat. Tumor burden was the percentage of total mammary tumor weight in a tumor-bearing rat relative to the host body weight, which was the final body weight less the total mammary tumor weight. Tumor latency was the average time that the first mammary tumor was palpated in a tumor-bearing rat in each dietary treatment group. Total tumor was the sum of palpable and non-palpable tumors. Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high-zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).
2 Total tumor was the sum of palpable tumors and non-palpable tumors.

a Significantly different from Z12 group (p < 0.05).

b Significantly different from Z155 group (p < 0.05).

c Significantly different from PZ12 and PZ155 groups (p < 0.05).

d Significantly different from their corresponding ad libitum control group (PZ12 vs. Z12; PZ155 vs. Z155; p < 0.05).
Table 2-5. Effect of dietary zinc intake on the size of N-methyl-N-nitrosourea-induced mammary tumors

<table>
<thead>
<tr>
<th></th>
<th>Dietary treatments</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z3</td>
<td>PZ12</td>
<td>Z12</td>
<td>PZ155</td>
<td>Z155</td>
</tr>
<tr>
<td>Tumor volume (mm³)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>274 ± 82&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>572 ± 234</td>
<td>999 ± 218</td>
<td>428 ± 136&lt;sup&gt;c&lt;/sup&gt;</td>
<td>705 ± 121</td>
</tr>
<tr>
<td>Median</td>
<td>82</td>
<td>142</td>
<td>189</td>
<td>117</td>
<td>226</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.40 ± 0.13&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.72 ± 0.29</td>
<td>1.43 ± 0.36</td>
<td>0.65 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.93 ± 0.20</td>
</tr>
<tr>
<td>Median</td>
<td>0.13</td>
<td>0.21</td>
<td>0.31</td>
<td>0.14</td>
<td>0.30</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significantly different from Z12 group (p < 0.05).

<sup>b</sup>Significantly different from Z155 group (p < 0.05).

<sup>c</sup>Significantly different from their corresponding ad libitum control group (PZ12 vs. Z12; PZ155 vs. Z155; p < 0.05).

<sup>1</sup>Values represent the mean ± SEM. Twenty-five rats were assigned to each dietary treatment group, except Z3, PZ155 and Z155, where there were 33, 23, and 24 rats, respectively, in each group. Tumor volume was calculated by using the following formula: \( w \times h \times l \times \pi/6 \). Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high-zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).
Table 2-6. Effect of dietary zinc intake on the type of N-methyl-N-nitrosourea-induced mammary tumors

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Dietary treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z3</td>
</tr>
<tr>
<td>Comedo</td>
<td>0</td>
</tr>
<tr>
<td>Ductal</td>
<td>3</td>
</tr>
<tr>
<td>Grade 1 Papillary</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2 Papillary</td>
<td>9</td>
</tr>
<tr>
<td>Grade 2 Papillary and Comedo</td>
<td>0</td>
</tr>
<tr>
<td>Grade 2 Papillary and Cribiform</td>
<td>3</td>
</tr>
<tr>
<td>Cribiform</td>
<td>2</td>
</tr>
<tr>
<td>Total Tumors</td>
<td>18</td>
</tr>
</tbody>
</table>

1 The dietary treatment groups were Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high-zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).
Table 2-7. Effect of dietary zinc intake on tumor grade of N-methyl-N-nitrosourea-induced mammary tumors

<table>
<thead>
<tr>
<th>Tumor Grade</th>
<th>Dietary treatments</th>
<th>Z3</th>
<th>PZ12</th>
<th>Z12</th>
<th>PZ155</th>
<th>Z155</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade</td>
<td></td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>6</td>
<td>5</td>
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<td>High grade</td>
<td></td>
<td>17</td>
<td>33</td>
<td>27</td>
<td>26</td>
<td>28</td>
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</tbody>
</table>

1The dietary treatment groups were Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high-zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).
Figure 2-1. Effect of dietary zinc intake on the cumulative incidence of palpable mammary tumor in N-methyl-N-nitrosourea treated rats. The tumor incidences among dietary treatment groups in each week not sharing a common letter are significantly different ($p<0.05$). There was no statistical analysis done on week 6 and 7 data as the expected value for Chi square analysis was insufficient. Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high-zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).
Figure 2-2. Effect of dietary zinc intake on the cumulative number of palpable mammary tumors in N-methyl-N-nitrososurea (MNU) treated rats. Z3: low-zinc diet (3 mg zinc/kg diet); PZ12: pair-fed with-adequate-zinc diet (12 mg zinc/kg diet); Z12: adequate-zinc (12 mg zinc/kg diet); PZ155: pair-fed-with-high-zinc diet (155 mg zinc/kg diet); Z155: high-zinc (155 mg zinc/kg diet).
Weeks Post MNU Injection

Cumulative Number of Palpable Mammary Tumors

- Z3
- PZ12
- Z12
- PZ155
- Z155
Figure 2-3. Grade 2 papillary carcinoma characterized by sparse fibrovascular core (F), serrated luminal border (S) and epithelium cell layers greater than 5-10 cell layers thick. (Hematoxylin and Eosin)
Figure 2-4. Transition between Grade 1 papillary carcinoma with cribiform pattern (upper half) and fibroadenoma (lower half). (Hematoxylin and Eosin)
5. REFERENCES


CHAPTER 3
GENERAL DISCUSSION AND CONCLUSIONS

1. MODEL OF RAT MAMMARY TUMORIGENESIS FOR HUMAN BREAST CANCER

Induction of mammary tumorigenesis in 50-day-old Sprague-Dawley rats using N-Methyl-N-nitrosourea (MNU) better simulates human breast cancer than induction with DMBA because it produces tumor invasion, metastasis and a lower proportion of benign lesions. As mentioned earlier, greater than 85% of MNU-induced rat mammary tumors harbor the \(H\)-ras mutation (Sukumar et al., 1983; Zarbl et al., 1985), but in human breast cancer \(ras\) gene mutations are never or rarely seen. Mutation of \(ras\) gene is more commonly found in tumors of the pancreas (90%), colon (50%), lung (30%), thyroid (50%) and myeloid leukemia (30%) (Bos, 1989). von Lintig (2000) reported that 5% of breast cancer harboring \(ras\) gene mutations. Even though \(ras\) mutations are not common in breast cancer, it may be activated by over expression of growth factor receptors that signal through the \(ras\) oncogene such as EGF and Erb-B2 receptor (von Lintig, 2000). Animal models provide an understanding of the initiation, promotion and progression of mammary tumors, but extrapolation of the results to humans should be cautious.

2. EFFECTS OF DIETARY ZINC INTAKE ON MAMMARY TUMORIGENESIS

Zinc is known as a regulator of apoptosis as zinc depletion in cultured breast cancer cells induces apoptosis (Sunderman, 1995; Wu, 2003). Zinc deprivation inhibits DNA synthesis and cell proliferation in cultured cells (Chesters et al., 1989; Prasad et al., 1996; MacDonald et al., 1998; Paski and Xu, 2001, 2002) and in rats (James et al., 1987; Lawson...
et al., 1988). This could theoretically result in a lower number of initiated cells with the H-
ras gene mutation, fewer neoplastic cells, and ultimately, lower tumor incidence. This study
provided some evidence to support this concept.

In this study, there was no difference on all the endpoints used to assess MNU-
induced mammary tumorigenesis in Z12 rats vs. Z155 rats and PZ12 rats vs. PZ155 rats,
suggesting that zinc supplementation had no effect on MNU-induced rat mammary
tumorigenesis (Table 2-4 and 2-5). This lack of an effect of zinc supplementation on MNU-
induced mammary tumorigenesis perhaps reflects the fact that increasing zinc concentration
beyond requirement does not further enhance growth. Moreover, zinc supplementation
beyond dietary recommendation level does not increase tissue zinc concentration due to zinc
homeostasis. During the periods of high zinc intakes, zinc absorption is decreased while zinc
excretion is increased (King, 1990). Woo (1999) reported that feeding rats with a diet
containing 171 mg zinc/kg diet had no effect on all the tissues tested, including femur, liver,
skin, small intestine, lung, muscle, heart, and mammary glands, compared with that in rats
fed a diet containing 31 mg zinc/kg diet.

3. EFFECTS OF DIETARY ZINC INTAKE ON HISTOLOGICAL
CHARACTERISTICS OF MNU-INDUCED RAT MAMMARY TUMORS

Although MNU-induced rat mammary tumorigenesis was affected by dietary zinc
intake and feed intake level, the tumor grade was not affected by either dietary zinc intake
levels or feed intake level. Moreover, dietary zinc intake also did not have an effect on the
histological characteristics of the tumors. The carcinogenicity of MNU is due to its ability to
alkylate nucleotides causing mutation of H-ras oncogene. Once the DNA lesion is initiated,
the initiated cells will undergo DNA repair, cell death or cell division under the right growth environment, such as the right hormonal levels and nutrients, to form neoplastic cells. Theoretically, a higher number of neoplastic cells will lead to higher incidence and number of tumors. Thus, a lower tumor incidence, tumor number, and tumor multiplicity in Z3 rats suggests that low zinc may influence the initiation and promotion phases of MNU-induced rat mammary tumorigenesis. On the other hand, tumor behavior, such as benign vs. malignant, and tumor grade, reflects the genetic makeover of the tumor. Collectively, the results reported herein suggest that although dietary zinc intake had an influence on MNU-induced tumorigenesis, it did not have an influence on the genetic control of tumor behavior.

MNU-induced mammary tumorigenesis in this study resulted in only malignant tumors, which was in agreement with an earlier report. Thompson et al. (1998) also reported a lack of benign tumors in MNU-induced mammary tumors. However, several other studies showed the presence of both benign and malignant mammary tumors in MNU-treated rats (McCormick et al., 1981; Kumar et al., 1990; Thompson et al., 1992). This difference could be because benign tumors have a longer latency period compared to malignant tumors (McCormick, 1981). Presence of benign tumor per rat was observed at approximately 140 days after MNU induction, but in this study rats were kept for only 98 days post-MNU.

4. OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

In the present study, 50-day-old sexually mature female Sprague-Dawley rats on different dietary zinc treatments were injected with the mammary carcinogen MNU. This study found that feed intake and body weight gain in the Z3 rats was significantly lower than the Z12 and Z155 rats ($p<0.05$), whereas weight gain was similar to PZ12 and PZ155 rats.
The plasma zinc level in the Z3 rats was only 50% of that in the pair-fed and ad libitum control rats. The incidence of palpable and total tumor number in the Z3 rats was significantly lower than all the control groups ($p<0.05$). The number of palpable and total mammary tumors per tumor bearing rat was also lower in the Z3 rats compared to the control groups. Tumor burden in the Z3 rats was significantly lower than in the Z12 and Z155 rats ($p<0.05$), but not different the PZ12 and PZ155 rats. Tumor latency was significantly longer than that in the Z12 and Z155 rats ($p<0.05$), but was the same as in the PZ12 and PZ155 rats. Tumor volume and tumor weight in the Z3 rats were significantly lower than that in the Z12 and Z155 rats ($p<0.05$), but not different from the PZ12 and PZ155 rats. Dietary zinc intake did not have an effect on the tumor grade. This may have been due to the effect of low zinc on cell proliferation. The effects of dietary zinc intake on MNU-induced mammary tumorigenesis in the present study showed that marginal low zinc intake exerted some suppressive effects on MNU-induced rat mammary tumorigenesis.

Lower host body weight gain in the Z3 rats compared with the Z12 and Z155 rats was due to reduced zinc and feed intake. The lower multiplicity of palpable tumors, tumor latency, tumor size and tumor burden seen in the Z3 rats were caused by reduced feed intake associated with low zinc intake.

In future studies, the body plasma zinc concentration should be determined at more time points during the course of mammary tumorigenesis. This information could provide a better assessment of the roles of zinc in MNU-induced rat mammary tumorigenesis. It was unfortunate that the plasma zinc levels were not assessed at the time of MNU injection to ensure the zinc deficiency status of the Z3 rats, rather than totally rely on monitoring feed intake and the appearance of cyclical pattern of feed intake and zinc deficiency signs.
Common alkylation products, such as O\textsuperscript{6}-methylguanine and 7-methylguanine, are detected in mammary glands soon after MNU injection (Cox and Irving, 1979). Both of these products are removed by O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT). If these products are not removed, they can induce DNA lesions in the second nucleotide of codon 12 in the H-ras oncogene (Zarbl et al., 1985). Once the mutation is initiated, the cell will undergo rapid proliferation provided there are enough nutrients required for growth. To determine the effects of dietary zinc intake on tumor initiation, it would be valuable to assess whether zinc has an effect on MGMT levels and activity. DNA repair is an important event in the initiation of cancers. DNA lesions within the H-ras mutation that are not repaired can lead to continual cell growth as an activated H-ras gene can stimulate growth in the absence of regulating factors (Engelbergs et al., 2000). Fong et al. (1988) showed that methyltransferase activity was significantly reduced in the esophagus, spleen and lungs in zinc deficient rats compared to the control rats, resulting in a higher susceptibility to carcinogens.

However, even though MNU induces mutations in the H-ras oncogene, it may not lead to clonal expansion of the initiated cell stimulated by gland development. Lu and Archer (1992) reported that Copenhagen rats resistant to mammary tumor induction by MNU resulted in H-ras mutations in the epithelial cells of the mammary glands. However compared to susceptible rats, there was a decreased number of cells containing the mutation. Therefore, even though there were initiated cells containing H-ras, these cells were unable undergo sustained clonal expansion. Lower tumor incidence and tumor number in Z3 rats than that in the control rats may indicate that marginal low zinc intake influences the number of initiated cells, clonal expansion or both. Zinc deficiency reduces DNA and protein syntheses because even though cells are stimulated to proliferate in the presence of the H-ras
oncogene, the amount of zinc required for this process is not met. In addition to the effects of an inadequate level of zinc for cell growth, the induction of apoptosis may be another reason for the lowered tumor incidence.

The estrus cycle of rats should be determined because rats have a higher rate of DNA synthesis on the day of proestrus. This results in significantly higher numbers of progressive mammary tumors, higher tumor growth rate, and a higher number and weight of tumors per tumor-bearing rat (Nagasawa et al., 1976). In future studies, the effects of marginal low zinc intake on estrogen receptors should also be investigated because estrogen is a growth factor in mammary tumors. Estrogen receptors (ER) are zinc finger proteins and a decrease in functional ERs could have an effect on growth. As well, it was concluded that feed restriction resulted in a change in the hormonal secretion (ThyagaRajan et al., 1993).

Finally, it is important to determine if marginal zinc deficiency is effective at reducing the risk of mammary tumorigenesis. Understanding the mechanism of how zinc deficiency influences tumor initiation, promotion and progression may allow for the development of pharmacological interventions to mimic the effects of zinc deficiency at the tumor site.
5. REFERENCES


APPENDIX 1

DIETARY FORMULATION
Table A-1. Zinc-free mineral premix.

<table>
<thead>
<tr>
<th>Minerals</th>
<th>Company</th>
<th>Catalogue number</th>
<th>g/kg mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Carbonate</td>
<td>Fisher</td>
<td>C64-3</td>
<td>83.56</td>
</tr>
<tr>
<td>Calcium Phosphate</td>
<td>Sigma</td>
<td>C-7263</td>
<td>376.4</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>Fisher</td>
<td>S271-50</td>
<td>65.00</td>
</tr>
<tr>
<td>Tri-Potassium Citrate</td>
<td>BDH</td>
<td>1020045</td>
<td>228.00</td>
</tr>
<tr>
<td>Potassium Sulfate</td>
<td>BDH</td>
<td>B10220</td>
<td>45.50</td>
</tr>
<tr>
<td>Magnesium Oxide</td>
<td>BDH</td>
<td>101504G</td>
<td>24.00</td>
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<tr>
<td>Ferric Citrate</td>
<td>Sigma</td>
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<td>6.06</td>
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<td>Manganese Carbonate</td>
<td>Aldrich</td>
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<td>Chromium Potassium Sulfate</td>
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<td>ICN</td>
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<td>167.182</td>
</tr>
<tr>
<td>Vitamin</td>
<td>Company</td>
<td>Catalogue #</td>
<td>g/kg mix</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>Thiamin HCL</td>
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<td>Riboflavin</td>
<td>ICN</td>
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<td>Pyridoxine HCL</td>
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<td>Nicotinic Acid (niacin)</td>
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<td>D-pantothenic Acid</td>
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<td>Folic Acid</td>
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<td>D-biotin (Vitamin H)</td>
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<td>Cyanocobalamin (0.1% in Manitol)</td>
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<td>Retinyl Acetate 500,00IU/g</td>
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<td>103257</td>
<td>0.80</td>
</tr>
<tr>
<td>Dl-alpha-tocopherol Acetate (250IU/g)</td>
<td>ICN</td>
<td>100555</td>
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<td>Cholecalciferol (4000,000 IU/g)</td>
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<td>Menadione (Vitamin K)</td>
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Table A-3. Zinc premix for dietary treatments

<table>
<thead>
<tr>
<th>Premix</th>
<th>ZnSO₄ · 7H₂O (g/kg mix)</th>
<th>Corn Starch (g/kg mix)</th>
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<tr>
<td>98-Z1</td>
<td>0.90</td>
<td>999.1</td>
</tr>
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<td>98-Z2</td>
<td>4.80</td>
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<tr>
<td>98-Z4</td>
<td>67.7</td>
<td>932.3</td>
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</tbody>
</table>

¹ZnSO₄ · 7H₂O contains 22.74% as zinc by weight. At 1% of the diet (w/w), 98-Z1, -Z2, and -Z4 provided 2, 11, and 154 mg of zinc to each kg of diet to the low-, adequate-, and high-zinc diet, respectively.
Table A-4. Modified AIN-93G egg white based diet

<table>
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<tr>
<th>Ingredient</th>
<th>Company</th>
<th>g/kg diet</th>
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<tbody>
<tr>
<td>Egg White</td>
<td>ICN</td>
<td>200.0</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>ICN</td>
<td>572.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>ICN</td>
<td>100.0</td>
</tr>
<tr>
<td>Soybean Oil(^1)</td>
<td></td>
<td>70.0</td>
</tr>
<tr>
<td>Mineral Premix</td>
<td></td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin Premix</td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>Sigma</td>
<td>2.5</td>
</tr>
<tr>
<td>Zinc premix</td>
<td></td>
<td>10.0</td>
</tr>
</tbody>
</table>

\(^1\)Soybean Oil from Canasoy Vancouver, BC. Without Zinc premix, this diet provides about 1mg Zn/kg diet
APPENDIX 2

HISTOLOGICAL ORGANIZATION OF N-METHYL-N-NITROSOUREA-INDUCED RAT MAMMARY TUMORS
Figure A-1. Normal and Grade 1 papillary carcinoma. A) Normal mammary glands characterized by ducts lined with two cell layers comprising inner epithelial layer and outer myoepithelial layer (arrow). The ducts are surrounded by loose connective tissue (CT), adipose tissue (F) and blood vessels (BV). Smaller ducts (SD) are also shown in this figure. B) Grade 1 papillary carcinoma characterized by prominent fibrovascular cores (F) covered by epithelium cell layers less than 3 cell layers thick with smooth luminal border (arrow). C) Higher magnification of Grade 1 papillary carcinoma, in which mitotic figures are scarce and nuclear pleomorphism is moderate (arrow). (Hematoxylin and Eosin)
Figure A-2. Grade 2 papillary carcinoma with thickened epithelial cell layer and absence of a fibrovascular core, pleomorphic nuclei, prominent nucleoli (P) and frequent mitotic figures (arrow) at higher magnification. (Hematoxylin and Eosin)
Figure A-3. Comedo, cribiform and ductal carcinoma. A) Comedo carcinoma characterized by distended ductal structures lined by multilayered epithelial cells surrounding necrotic debris (N). B) Cribiform carcinoma characterized by solid sheets of neoplastic epithelial cells with various sized punched out lumens (C). C) Ductal carcinoma characterized by epithelial cells growing inwards (arrow) forming papillary and cribiform patterns devoid of a fibrovascular core. Proteinaceous material (P) is present in the lumen of the ducts. (Hematoxylin and Eosin)
Figure A-4. Grade 2 papillary carcinoma with cribiform areas.
Figure A-5. High grade tumor with invasion. A) High grade tumor showing invasion into skeletal muscle (M) with inflammation and reaction to invasive areas (arrow). B) High grade tumor showing adipose tissue invasion (F) with resulting inflammation and reaction of surrounding tissues (arrow). C) High grade tumor showing perineural invasion. Carcinoma cells are adjacent to nerve bundle (N). (Hematoxylin and Eosin)
Figure A-6. Low grade *in situ* and invasive carcinoma. A) Low grade *in situ* carcinoma showing one to two layers of epithelial cells (arrow) surrounding the duct with minimal inflammation. B) Low grade invasive carcinoma showing invasion into skeletal muscle (M) with prominent inflammation (I) in the surrounding area. (Hematoxylin and Eosin)