THE EFFECTS OF PROPHYLATIC PROTOCOLS OF EXERCISE AND TAMOXIFEN ON THE INDUCTION AND GROWTH OF ESTROGEN DEPENDENT AND INDEPENDENT RAT MAMMARY TUMORS.

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Master of Science in Biology

by

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6-12-96
Date
With breast cancer affecting one out of every nine women in the United States in their lifetime and a 50% mortality rate among those who have the disease, efforts have been initiated to find a prophylactic regimen which would decrease a women's chances of developing breast cancer. The central purpose of this study was to characterize the influence of moderate levels of exercise on the induction and development of estrogen dependent and independent rat mammary tumors. This characterization was compared against the known antiestrogenic effects of tamoxifen. The tumor model used was 7,12-dimethylbenz(a)anthracene (DMBA) induction of rat mammary cancer. Hormone dependency was determined by observing tumor growth following oophorectomy. Although the results of the experiments yielded no indication of tumor inhibition due to the exercise
Regimen, rats treated with tamoxifen showed a significant reduction in body weight and mammary tumorigenesis. In the control group, an equal number of hormone-dependent and hormone-independent tumors were seen following removal of the ovaries. In the exercise group, a selection against hormone-independent tumors was observed.

Accepted by: [Signature]

Chair
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INTRODUCTION

With one out of eight women developing breast cancer, mammary carcinoma is the most common form of cancer affecting women in the United States. Since 1980, there had been a steady 2% increase in the incidence of breast cancer, but recently the incident rate has leveled off at approximately 182,000 new cases per year. The prevalent use of mammography for early detection during this period is believed to be responsible in part for the rise. The balance of cause for the rise remains unknown. Approximately 46,000 women per year will die from a metastatic progression of the disease (American Cancer Society 1994).

In light of the inability to lower the mortality rate, attention and effort of scientists and clinicians has turned toward reduction of the incidence of breast carcinomas (Bernstein et al., 1992; Kelsey 1993). New prognostic techniques (Gail et al., 1989) and the discovery of the breast cancer linked genes, BRCA1 and BRCA2, (Miki et al., 1994; Furteal et al., 1994; Wooster et al., 1994) have facilitated the possibility that women with a higher individual risk could be targeted for prophylactic treatment.
A proposed intervention strategy involved the use of the adjuvant therapeutic agent tamoxifen, which had been successfully used to prevent tumor recurrence in breast cancer patients (NATO, 1985). Tamoxifen, a non-steroid antiestrogen, blocks the stimulatory effects of estrogen, which is considered the primary promoter in estrogen-dependent and early, estrogen-independent tumor cell lines. Due to tamoxifen's demonstrated ability to suppress recurrence in cancer patients, the Breast Cancer Prevention Trial was initiated to compare benefits of tamoxifen's prophylactic use versus the possible side effects in women who are assessed to have a high risk of developing breast cancer (Bush & Helzlsouer 1993; Kelsey, 1993; NSABP, 1992).

An alternate strategy of prevention has been a proposed alteration in lifestyle factors associated with breast cancer. Changes in components of everyday life, such as diet and exercise, have been the focus of many researchers because a concern over the ramifications of sustained hormonal manipulation (Bernstein et al, 1993). Exercise has received attention because laboratory and epidemiological evidence has shown an associated reduction in breast cancer incidence. Despite this evidence, little is known about the cause and effect of
the relationship between exercise and breast cancer incidence.

Breast Cancer

Breast carcinomas originate from the transformation of the epithelial cells which line the lobules and ducts of the breast. Seventy to eighty percent of breast tumors are ductal in origin (American Cancer Society, 1987).

Transformation occurs when the cell’s genome is altered resulting in a failure of the mechanisms which regulate the cell cycle. In order for a tumor to develop from a single transformed cell, cell division must occur. Each cell cycle results in the passing on the altered chromosome, compounding the damage to the chromosome and/or altering chromosomal distribution. Cell division in tissues like the mammary epithelium is influenced by endogenous hormones or exogenous analogs. Epidemiological observations, such as the increased risk associated with early menarche, late first full-term pregnancy, and the use of postmenopausal estrogen replacement therapy, indicate that ovarian hormones have a central role in the initiation and promotion of breast cancer (Am. Red Cross, 1994). Menstrual cycling and
pregnancy events are marked by large changes in the ovarian hormones estrogen and progesterone (Berstein & Ross, 1993).

Growth of the mammary tissue occurs during two hormone dependent events, puberty and pregnancy. Although both processes are stimulated by estrogen, progesterone, insulin, cortisol, and prolactin, the timing and concentration of exposure to these hormones result in the distinct changes occurring between the events. Estrogen has a key role in puberty where it potentiates the action of prolactin and its concentration directly influences the growth and development of mammary tissue (Speroff et al., 1994). In the animal models, estrogen has been demonstrated to have a direct effect on mammary epithelial cell development by stimulating growth and branching (Silberstein et al., 1994; Halsam, 1988; Daniel et al., 1987).

Estrogen- and progesterone-dependent changes in mammary tissue occur during normal menstrual cycles. During late luteal phase, stimulation by elevated levels of estrogen and progesterone results in maximal breast size. Increases in fluid secretion, mitotic activity and gene expression of nonglandular tissue and glandular
epithelium mark this period of size increase in the luteal phase (Speroff et al., 1994).

The predominant estrogens circulating in the blood are estradiol-17β and estrone. Estradiol-17β has been demonstrated to be the most biologically active form of estrogen in mammary tissue (Pasqualini, et al, 1991). During premenopausal years, almost all estrogen is ovarian in origin with estradiol-17β in higher plasma concentrations than estrone. After menopause, direct production of estrogen by the ovaries ceases. Most postmenopausal estrogen arises from the aromatization of adrenal androgens to estrone. In turn, some estrone is metabolized into estradiol. The postmenopausal plasma concentrations of estrone are significantly higher than estrogen (Bernstein & Ross, 1993).

Mammary tumors are normally characterized in part by estrogen dependence or independence. Measurements of the level of estrogen receptors in tumors are used in prediction of patient prognosis and viability of antiestrogen adjuvant therapy. Though clinical assays can produce qualitative and quantitative measurements of tumor ER, tumor hormone sensitivity does not correlate precisely with estrogen receptor status. Over 60% of human breast tumors are estrogen receptor positive (ER+)
and only two-thirds of these ER+ tumors are expected to be controlled by antiestrogen therapy. Further, 5-10% of estrogen receptor negative (ER-) assayed tumors have proven to be sensitive to antiestrogen therapy (Pentrangeli et al., 1994; McClelland et al., 1987; McClelland et al., 1986; Osborne et al., 1980; DeSombre & Jensen, 1980; Paridaens et al., 1980; Wittliff et al., 1980). Despite the discrepancy between ER status and estrogen sensitivity, the value of the ER assay was elucidated by the relatively low percentage of ER+ cells in normal tissue, which is less than 20 percent (Ricketts et al., 1991; Peterson et al., 1987). Pentrangeli et al. (1994) studying breast carcinoma biopsies found a significantly higher percentage of ER+ positive cells in neoplastic tissues (75%) compared to perineoplastic tissue (57%), and that there was a positive association between ER expression and ER gene hypomethylation. Normal methylation of the estrogen receptor gene can inhibit the expression of the receptor. The elevated expression of the ER in neoplastic tissue indicates the significance of estrogen in the pathogenesis of most breast cancers.

Through interaction with the estrogen receptor, estrogen mediates many cellular activities essential for
tumor growth. Estrogen triggers DNA synthesis and gene transcription in normal and cancerous mammary cell lines. Release of growth stimulating, autocrine and paracrine polypeptides, such as transforming growth factor-α, epidermal growth factor (Bates et al., 1988; Imai et al., 1982), insulin-like growth factor-II (Brunner et al., 1993; Osborne et al., 1989) and platelet-derived growth factor (Rosengurt et al., 1985.), has been shown to be increased with estrogen stimulation. Conversely, a reduction in the levels of the inhibitory factor, transforming growth factor-β was affected by estrogen in the tumor cells (Knabbe et al., 1987).

Animal studies have demonstrated that estrogens have a positive influence on the induction and promotion of mammary tumors in animal studies and that reversal of this influence can be achieved by oophorectomy or treatment with antiestrogens (Doa, 1981; Jordan, et al., 1990; Jordan et al 1980).

Epidemiological studies have shown that breast cancer risk increased in women having frequent, relatively short menstrual cycles (Pike et al., 1993; Whelan et al., 1992; Henderson et al., 1985). Women with these frequent ovulatory cycles are believed to be at higher risk because more of their reproductive years
are spent in the luteal phase than women having less frequent ovulatory cycles. The high mitotic activity that marks the luteal phase is considered to be the source of the elevated risk. Consequently, women who experience anovulation or amenorrhea are shown to have a reduced risk of mammary cancer (Pike et al., 1993; Whelan et al., 1992).

Mechanism of Steroid Action

The mechanism of steroid action can be demonstrated by the pathway of agonism of the estrogen hormones (Figure 1). Estrogen influences the activity of the target cell through a mechanism centered around the nuclear, estrogen receptor (ER). The hydrophobic, estrogen molecule is carried to mammary tissue by a sex hormone binding globulin (SHBG). The estrogen molecule becomes biologically active by dissociating from SHBG and diffusing through the lipid bilayer of the plasma membrane. The steroid passes through the cytosol and transverses the nuclear membrane. In the nucleus, the estrogen binds to the ER altering the conformation of the receptor (Figure 1). This conformational change causes the release of a heat shock protein and activates the receptor. The activated ER-estrogen complex then
dimerizes with another activated ER-estrogen complex. The dimer binds to the estrogen response element (ERE) on the target chromosome. Dimer binding to the ERE and interaction with RNA polymerase promotes the transcription of the estrogen-mediated gene downstream. The products of these genes potentiate the actions associated with estrogen stimulation (Tsai and O’Malley, 1994).
Figure 1: Mechanism of steroid action modified from Tsai and O’Malley (1994). SHBG=sex steroid binding globulin, HSP=heat shock protein, mRNA=messenger ribonucleic acid, DNA=deoxyribonucleic acid.
Tamoxifen

Tamoxifen (Fig. 2), \([Z]-2-[4-(1,2\text{-}diphenyl\text{-}1\text{-}butenyl)}\text{-}\text{phenoxy}]\text{-}N,N\text{-}\text{dimethylethanamine},\) is a non-steroid, antiestrogen first synthesized in 1966 (Harper MJK & Walpole AL, 1966). During the 1970s, it was discovered that tamoxifen demonstrated antiestrogenic and

\[
(CH_3)_2N(CH_2)_2O \quad \text{R}
\]

Figure 2: Tamoxifen molecule. Functional group \(R\) represents hydrogen in the basic molecule and a hydroxyl group in trans 4-hydroxy-tamoxifen.
antitumor behavior in rat models (Jordan, 1974; Cole et al., 1975; Nicholson & Golder, 1975; Jordan et al., 1978) and humans (Cole et al., 1971). Since then, numerous trials have shown tamoxifen's ability to decrease cancer recurrence and patient mortality rates (Bush & Helzlsouer, 1993). In recent years, tamoxifen has been proposed as prophylactic breast cancer agent for use by women, who have family history or genetic disposition toward developing a mammary carcinoma.

The chemopreventative properites of tamoxifen have been demonstrated in animal models. Jordan et al, (1976) noted a reduced tumor number and increased time of appearance in a DMBA-induced mammary tumor model with use of tamoxifen. Parallel observations were made in the nitrosomethylurea (NMU)-induced mammary carcinoma model (Turcot-Lemay & Kelley, 1980). Long term tamoxifen use has been demonstrated to suppress tumor genesis (Jordan et al., 1991). Further, tamoxifen reduced by 90% the number of spontaneous tumors in a study of older rats (Maltoni et al., 1988).

Tamoxifen has been shown to be an effective antitumoral and chemopreventative agent in mammary tissue. Elucidation has shown that it is a cytostatic agent, not a cytotoxic agent (Jordan, 1994). Studies in
vitro with human cancer cells, such as the MCF-7 cell line, have shown that tamoxifen inhibits transition from the G₀-G₁ phase resulting in suppression of cell cycling. This cytostasis produces an increase in the number of cells in G₀-G₁ phase and a decrease in number of cells in S, G₂, and M phase in the cell cultures (Taylor et al., 1983; Sutherland et al., 1983; Osborne et al., 1983). Using $^{3}$[H]-thymidine assays, Osborne et al. (1992) showed that preneoplastic cell lines were less proliferative in an animal under a tamoxifen protocol. Additionally, removal of tamoxifen therapy from animals that have been transplanted with a cancer cell line resulted in the appearance of tumors (Jordan et al., 1991).

The cytostatic influence on cancer development when tamoxifen is used prophylactically may select against estrogen-responsive cells producing a higher percentage of the more aggressive, estrogen-independent tumors. Zimniski and Fendl (1992) studying animals receiving coincidental doses of tamoxifen and a carcinogen witnessed a reduction in the total tumor incidence and 100% selection of hormone-independent tumors. Sylvester et al. (1987) showed an 3-fold increase in the percentage of estrogen-independent tumors in a study with a prophylactic dosage of tamoxifen in a DMBA rat model.
With respect to tamoxifen's antitumoral properties, interaction with the estrogen receptor is central to the antiestrogens influence. While tamoxifen has a very low affinity for the ER, one of its metabolites, trans 4-hydroxy-tamoxifen (Figure 2), has an affinity for the ER nearly equal to that of estradiol (Kemp et al., 1983). The binding of the receptor by the metabolite blocks the agonistic activities associated with normal estrogen-ER interaction (Jordan et al., 1977; Allen et al., 1980; Borgna & Rochefort, 1981). Molecular analysis has shown that the tamoxifen molecule fits into the estradiol binding pocket on the ER. The pocket contains a number of hydrophobic residues and a glutamate residue. The phenolic group of 4-hydroxytamoxifen occupies the same position relative to the hydrophobic groups as the A-ring of estradiol, but the glutamate residue is unable to hydrogen bond with the antiestrogen molecule. The long nitrogen-containing side chain inhibits interaction between arginine and aspartate residues of the ER necessary for its activation (Lewis et al., 1995). This positioning in the ER blocks estradiol binding without activating the receptor. Estrogen-related gene activation is blocked by the tamoxifen-ER complex, which is not capable of dimerizing with other activated
complexes and blocking estrogen-ER mediated cell stimulation.

Exercise

Exercise has been demonstrated to have beneficial effects on a number of disease processes, such as those of cardiovascular disease, diabetes, osteoporosis, and colorectal cancer (Friedenreich and Rohan, 1995). These observations have raised a debate as to whether or not exercise would have an influence on the processes that govern the etiology of cancer. The influence of exercise on the disease processes of breast cancer is believed to be due to the 1) alterations in the energy balance, 2) endocrine system effects; and 3) stimulation of the immune system (Thompson 1992). The influence on breast cancer has been studied in both animal and epidemiological studies.

Exercise is physical activity specifically designed to improve physical fitness. There are three primary components of physical activity: duration, the length of the exercise bout; intensity, the work-rate of the exercise bout; and frequency. Aerobic exercise is characterized by a duration of at least 15 to 20 minutes; a frequency of at least 3 to 4 times a week; and an
intensity that will lead to improvement of aerobic capacity. Aerobic capacity can be measured by an improvement in maximal oxygen consumption, \( V_{O_2}^{\text{max}} \) (Caspersen, 1989).

In the rodent model, the effect of exercise during tumor initiation (commonly considered to be the first seven days after carcinogen administration (Thompson, 1994) and/or promotion phases of tumor development have been tested. Exercise begun during the initiation phase has been shown to decrease tumor incidence (Yedniak et al., 1987; Sakamoto et al., 1992; Moore and Tittle, 1973) or have no effect (Thompson, 1994). Studies with exercise protocols beginning during the promotional phase have yielded either decreases (Cohen et al., 1992; Bennick et al., 1986; Cohen, et al., 1988; Thompson, 1994; Thompson et al., 1995) or increases (Thompson et al., 1988; Thompson et al., 1989) in the proportional incidence of tumors in the animals. The discrepancy in experimental results may be due to differences in protocols with respect to duration and intensity. Energy balance, endocrine, or immunological alterations may be subject to the demands of the protocol.

Caloric expenditure of an exercise bout is a function of the duration and intensity of the activity.
As the caloric expenditure rises, the greater the effect it has on the body's energy balance. Thompson (1992) demonstrated a negative relationship between caloric-expenditure and tumor incidence. However, this assertion is challenged by the fact that in two studies Thompson et al. (1988; 1989) found no significant changes in carcass size or composition. Additionally, the purely caloric basis theory was confounded by the experiments of Thompson et al. (1995), which demonstrated a link between intensity and reduced tumor incidence. In that study carcinogenesis inhibition required an intensity that was 70% maximal and had no correlation with caloric expenditure. This last study suggests that there is an intensity threshold where caloric expenditure has a greater influence on mammary carcinogenesis (Thompson et al., 1995).

Another explanation for the discrepancy between the studies may be related to the altering of endocrine function. Thompson et al., (1995) stated that the duration of exercise had a greater effect than intensity on lengthening the animals' estrous cycle. A study using exercised rats which monitored varying intervals, indicated that extensive exercise induced changes in the frequency of release and strength of gonadotropin
releasing hormone and lutenizing hormone leading to amenorhea (Manning and Bronson, 1991). The expected luteal dysfunction or amenorhea resulting from the changes in the release patterns would lead to a significant reduction in levels of estrogen stimulating the mammary tissue and carcinomas.

Most epidemiological studies in humans have generated results favoring the role of exercise or high levels of physical activity in the reduction of breast cancer risk, but contradictory evidence does exist. A study of the alumnae of 10 American colleges showed a decreased risk for athletes when compared to non-athletes (Fisch et al., 1987). Other studies focusing on the breast cancer risk when compared with the level of caloric expenditure showed a decreasing incidence in breast cancer as the caloric demand of exercise increased (Bernstein et al., 1994; Friedenreich and Rohan, 1995). Further studies have shown that women in occupations with a high level of physical activity also benefited from a lowered incidence of breast cancer (Zheng et al 1993; Vena et al., 1987). In opposition to these observations, the Farmingham Heart Study (Dorgan et al., 1994) and the American Cancer Society's Cancer Prevention (Garfinkel and Stellmanm, 1988) cohort studies observed an
increased risk of breast cancer with increased physical activity.

During reproductive years, exercise has been shown to alter the pattern of regular menstrual cycling leading to a reduction in breast cancer incidence. Prospective studies where menstrual cycling characteristics were studied before and after training, demonstrated that exercise, especially strenuous exercise, can induce anovulation and luteal dysfunction, oligomenorrhea, and amenorrhea (Greene, 1993; Loucks, 1990; Keizer and Rogol, 1990). These interruptions of the menstrual cycle are believed to be the result of alterations in the hypothalamic-pituitary-ovarian axis, which lead to changes in levels and pulsatile release of GnRH and LH (Thompson, 1992; Greene, 1993; Loucks, 1990; Keizer and Rogol, 1990).

Problem Statement

The objective of this study was to establish the influence of a moderate exercise regimen on the induction and growth of 7,12-dimethylbenz(a)anthracene induced, estrogen-dependent and independent rat mammary tumors. Parallel protocols involving the use of tamoxifen served to assess the reduction of tumor incidence and influence
on estrogen dependence of exercise. Estrogen dependence was determined by changes in tumor volume preceding and following oophorectomy.

**Hypothesis**

Prophylactic regimens of exercise and tamoxifen will inhibit the induction and growth of mammary tumors induced by the introduction of 7,12-dimethylbenz(a)anthracene (DMBA). Specifically, exercise will have a broad inhibition on both estrogen-dependent and independent tumor cells; tamoxifen will have an influence on tumor growth and development with a greater effect on the estrogen-dependent tumors; and the two regimens in combination will have an additive effect on the inhibition of mammary tumor induction and growth.
METHODS AND MATERIALS

Animals

Forty-two female, Sprague Dawley rats (Harlan Sprague Dawley, Inc.) between 32-34 days of age, were housed two per cage in the Lappin Hall Animal Care Facility. They were placed on a 12 hour light/ 12 hour dark photoperiod. The rats were fed Purina Rodent Chow (Ralston Purina Company) and water ad libitium. The animals were maintained in the Lappin Hall Animal Care Facility.

Prior to the experiment, the animals were randomly divided and segregated into four groups: Group I-10 rats in the control group; Group II-11 rats in the exercise group; Group III-10 rats in the tamoxifen group; and Group IV-11 rats in the tamoxifen & exercise group. Group I was administered both the exercise sham and tamoxifen sham protocols. Group II underwent the moderate exercise and sham tamoxifen protocols. Group III received the experimental tamoxifen regime and sham exercise. Group IV was maintained on active exercise and tamoxifen protocols (Figure 3.)
Figure 3: Flow chart of research protocol by day. 1. Begin exercise protocols. 2. Begin tamoxifen protocols. 3. DMBA administration. 4. Oophorectomy. 5. Sacrifice.
Exercise

Rats of the Group II and Group IV were placed on a moderate exercise regimen (Thompson, et al, 1989) ten days before the introduction of carcinogen. The animals were exercised on a motorized treadmill (Lafayette Instruments, Inc.) using electric shock as motivation. The established regimen consisted of five, 15-minute exercise bouts per week with a belt speed of 20 meters per minute and inclination of 1°. To introduce the animals to treadmill running, the first five days of the experiment were marked by increasing speed and duration: day one (day -10 with respect to carcinogen introduction) at 8 meters/minute, 3-5 minutes; day two at 12 meters/minute, 10 minutes; day three at 12 meters/minute, 15 minutes; days four and five at 16 meters/minute for 15 minutes. The animals were run during the light period to insure that the activity was extracurricular to normal activity. This protocol was continued to the day 100 when the animals were sacrificed (Fig. 3).

Group I and Group III were maintained on sham treatment. The sham treatment involved placing the animal's cages into direct light for 15 minutes. This was done to equalize the stress of the control animals with those exercising on the unshaded treadmill.
**Tamoxifen**

The Group III and Group IV were started on a prophylactic regimen of tamoxifen citrate three days before carcinogen introduction. Each animal was administered 100 micrograms of tamoxifen citrate (Sigma Chemical Co.) in 0.1 ml peanut oil vehicle (Sigma Chemical Co.) five days a week (Osborne, 1992). After being suspended in a peanut oil vehicle using vigorous stirring, tamoxifen citrate was administered to the rats via subcutaneous injection using a 1.0 ml tuberculin syringe and 26 gauge needle. The tamoxifen protocol was continued until the animals were sacrificed (Fig 3).

Groups I and Group II were given subcutaneous injections of 0.1 ml of peanut oil on a schedule synchronous with the tamoxifen-administered groups.

**Carcinogen Administration**

On experimental day 0, the animals (age approximately 49-50 days) received 10 mg dose of 7,12-dimethylbenz(a)anthracene (Sigma Chemical Co.) dissolved in 1.0 ml of sesame oil (Sigma Chemical Co.). After light etherization, each animal was administered the DMBA via gastric intubation and injection with a 10 ml syringe.
Tumor Palpation

The date of carcinogen introduction was marked as day 0 with respect to the initiation of tumor development. Four weeks after the administration of DMBA introduction, the animals were checked daily for palpable tumors. Newly discovered tumors were recorded on the basis of individual rat identity, date of discovery and anatomical position.

Oophorectomy

Eighty-six days after carcinogen administration, an oophorectomy was performed on those animals with palpable tumors. All animals were anesthetized using 4 mg of sodium pentobarbital per 100 gm of body weight (Sigma Chemical Co.) dissolved in 0.1 ml distilled H₂O. The ovaries of tumor bearing rats (TBR) were removed via bilateral, dorsal incision. The animals were given four days to rest before resumption of tamoxifen and exercise regimens.

Tumor Measurement and Comparison

While anesthetized, the tumors of TBRs were measured using Vernier calipers. Measurements of the diameter
were taken for each tumor along three major axes and volumes calculated using the following equation: \( \text{Volume} = \frac{4}{3}\pi \text{Radius}_1 \text{Radius}_2 \text{Radius}_3 \). The calculated volumes were compared to ones taken two weeks later at sacrifice (Day 100). A decrease in tumor volume of greater than 20% over the two week period was given to indicate estrogen dependency (Fendl & Zimniski, 1992).

**Statistical Analysis**

Statistical evaluation of the experimental data were tested for significance using the Student's t-test at a level of significance of \( p<0.05 \).
RESULTS

Pre-Oophorectomy
Mean Animal Mass

Animals were weighed on day 86 just prior to oophorectomy. Comparison of the mean group masses showed a significant difference between the values for groups receiving the tamoxifen protocol and those that were receiving the peanut oil control (Figure 4). Larger mean masses were seen in the Group I (Control) and Group II (Exercise), which had 254g +/- 7.4g and 254g +/- 7.0g, respectively. Rats in Group III (Tamoxifen) and Group IV (Tamoxifen-Exercise) showed mean group masses of 211g +/- 7.7g and 203g +/- 6.4g. Cross comparisons using Student's t-test indicated a significant difference (p<0.05) between either Group I or Group II groups and Group III or Group IV.

Tumor Appearance

During the period between carcinogen introduction and oophorectomy, animals were examined for palpable tumors. Tumor appearance data for the experimental groups was characterized by a day-to-day progression in
Figure 4: Mean animal mass of the control, exercise, tamoxifen, and tamoxifen-exercise groups 86 days after DMBA administration.
number of tumor bearing rats (TBR) per group (Figure 5). Animals were added to the respective group's cumulative number of tumor bearing rats at the date of appearance of the first palpable tumor.

Group I yielded the most tumor bearing rats with six of ten animals having developed tumors during the observation period. The first palpable tumor appeared 47 days after carcinogen introduction. The remaining five animals had first tumors palpated at day 52; day 53, two rats; day 59 and day 73.

Five out of eleven animals palpated tumors in the Group II. First tumor appearance date for the exercise group was on day 53. The other four Exercise group tumor bearing rats palpated tumors on day 55, day 58, day 61 and day 70.

During the pre-oophorectomy observation period, the Group III had one tumor bearing rat in the experimental group of ten. This animal had the earliest palpable tumor appearance date, day 41.

The Group IV had one tumor bearing rat out of eleven animals. This tumor was palpated on day 45.
Figure 5: Control, exercise, tamoxifen, and tamoxifen-exercise rats with palpable tumors based on the day of first tumor appearance.
Group Tumor Information

Tumor data for experimental groups was accumulated for pre-oophorectomy period. Groups were compared on the basis of tumor bearing rats per group, average number of tumors per TBR, and total number of tumors (Table 1). There was found to be a significant difference in TBR per group between the either Group I or Group II and Group III or Group IV with respect to TBR per group.

Table 1: Group tumor data for pre-oophorectomy tumor bearing rats (TBR).

<table>
<thead>
<tr>
<th>Group</th>
<th>TBR per Group</th>
<th>Tumors per TBR</th>
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<tr>
<td>Control</td>
<td>6/10</td>
<td>2.17</td>
<td>13</td>
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<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise</td>
<td>5/11</td>
<td>1.20</td>
<td>6</td>
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<tr>
<td>(n=11)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tamoxifen</td>
<td>1/10&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.00</td>
<td>1</td>
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<td>(n=10)</td>
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<tr>
<td>Tam-Ex.</td>
<td>1/11&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.00</td>
<td>1</td>
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<tr>
<td>(n=11)</td>
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<sup>a</sup>=significant difference from the control group at p<0.05
<sup>b</sup>=significant difference from the exercise group at p<0.05
Post-oophorectomy

Group Tumor Information

Post-oophorectomy tumor data for the experimental groups were collected on the sacrifice date. The values were similar to that of pre-oophorectomy with a few exceptions (Table 2). The Group I had two tumors arise in an animal that had been oophorectomized. An unoophorectomized animal in the Group III developed a tumor on day 90. Finally, a loss of three animals while under anesthesia diminished the size of the Group II from eleven to eight animals. No significant differences were found with statistical analysis.

Table 2: Group tumor data for post-oophorectomy tumor bearing rats (TBR).

<table>
<thead>
<tr>
<th>TBR per Group</th>
<th>Tumors per TBR</th>
<th>Tumors per Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>6/10</td>
<td>2.50</td>
</tr>
<tr>
<td>Exercise (n=8)</td>
<td>2/8</td>
<td>1.00</td>
</tr>
<tr>
<td>Tamoxifen (n=10)</td>
<td>2/10</td>
<td>1.00</td>
</tr>
<tr>
<td>Tam-Ex (n=11)</td>
<td>1/11</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Estrogen Dependence and Independence

Determination of estrogen dependence or independence of tumors was done by a comparison of pre-oophorectomy tumor volume measurements with the post-oophorectomy volumes which were measured at sacrifice. Indication of estrogen dependence was established by a 20% decrease in volume over the two-week period (Fendl & Zimmiski, 1992). Tumors that appeared in the oophorectomized Group I animal were perceived to be estrogen independent because of their growth in an estrogen poor environment. The late arising tumor in the unoophorectomized Group III animal could not be classified. Additionally, no determination could be made for the tumors of the dead Group II animals.

Compiled estrogen dependence/independence data for the experimental group showed mixed results (Table 3). Group I animals were divided nearly equally between independent and dependent tumors. Even if the two late appearing tumors were disqualified, the division would still remain nearly equal with 7 dependent and 6 dependent tumors. With this trend in evidence, a fifty-fifty distribution between dependence and independence was used for assessment of the other tumor distributions.

Analysis of the Group II tumor distribution was significantly hindered by the loss of the three animals.
and their collective four tumors. The remaining animals showed a prevalence toward the development of estrogen dependent tumors (Table 3).

The Group III had just one tumor bearing rat. This rat had developed an estrogen dependent tumor, which seemed unusual in the presence of the antiestrogen tamoxifen.

The Group IV had a single tumor bearing animal with one tumor. The estrogen independent classification of this tumor met expectations in this tamoxifen-treated animal (Table 3).

**Table 3:** Assessment of estrogen dependence and independence of animal tumors.

<table>
<thead>
<tr>
<th></th>
<th>Estrogen Dependent Tumors</th>
<th>Estrogen Independence Tumors</th>
<th>Total Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Exercise (n=8)</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Tamoxifen (n=10)</td>
<td>1</td>
<td>0</td>
<td>2*</td>
</tr>
<tr>
<td>Tam-Ex (n=11)</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* One tumor developed in this animal that had not been oophorectomized.
Estrogen Dependence Vs. Appearance Time

Comparison of estrogen status, dependent or independent, with palpable-tumor appearance time yielded no observable trend. An examination of only the Group I distribution and appearance times also failed to yield any trend with respect to palpable appearance time and estrogen status.
DISCUSSION

The influence of exercise on the estrogen levels in the body and on the estrogen dependent/independent status of developing mammary tumors is largely unclear except for menstrual and ovarian cycle changes resulting from regimens involving intense, extended physical activity (Greene, 1993; Loucks, 1990; Keizer and Rogol, 1990). The estrogen dependent effects of moderate exercise levels remain uncharacterized.

Sustained exercise is considered to be an essential component in proper weight control programs, but in this study, the tamoxifen protocol not the exercise protocol was associated with a significantly lower body weight when compared to the controls (Figure 1). The observed lack of change in the exercise group body weight is consistent with other studies where no significant change in rat body weight and composition was seen in animals on similar exercise protocols when compared to controls (Thompson et al., 1988; Thompson et al., 1989). In looking at tamoxifen's effect of body weight, Wade and Heller (1993) observed that tamoxifen in dose-dependent fashion reduced body weight. They attribute this phenomena to a dose-dependent hypophagia; a decreased lipoprotein lipase activity, the blocking of estrogen receptors in adipose tissue, the associated reduction in
amount of adipose tissue; and as yet an uncharacterized interaction with the ER receptor in the brain and throughout the body. Examination of the body mass data from this study has indicated that tamoxifen's effects on body mass in the rat models are more pronounced than those of moderate exercise.

Tamoxifen's antiestrogenic and antitumoral properties led to its use in adjuvant breast cancer therapy and consideration for use in prophylaxis. The group tumor data demonstrated the antitumoral activity with the tamoxifen protocol producing a significant reduction in the number of tumor bearing animals within the respective groups when compared to the exercise and control groups (Table 1). In contrast to the antiestrogenic activity, a mixed result was seen in tumor dependence. As would be expected, the single TBR of Group IV (tamoxifen and exercise) developed an estrogen independent tumor, but the Group III (tamoxifen) TBR had one estrogen dependent tumor (Table 2). The dependent tumor's resistance to tamoxifen may be the result of estrogen receptor mutation, sequestering of tamoxifen by an antiestrogen binding sites, or metabolism of tamoxifen to a less antagonistic isomer (Osborne and Fuqua, 1994; Jordan, 1994; Pavlik et al., 1992). Development of
resistant tumors is one of the chief concerns in use of tamoxifen prophylaxis.

As the body adapts to exercise, marked changes in metabolism occur. One of the expectations of this study was to determine whether these adaptations have an influence on the tumor growth and induction. When compared to the control group data, the pre-oophorectomy numbers for the exercise group indicated that there was a decreased number of tumors per tumor bearing rat. However, in looking at the number of TBR per group or the first tumor appearance times of those tumors, the difference is less prominent. These differences in tumor pre-oophorectomy tumor data could be a result of altered hepatic metabolism associated with exercise which would effect the oxidase-dependent activation of the procarcinogen DMBA (Thompson, 1994; Dssing, 1985; Rosenblooom and Sutton, 1985).

Unfortunately, the loss of three out of five tumor bearing animals in exercise group compromised the most novel objective of this study, the determination of the influence of moderate exercise on the endocrine sensitivity of breast tumors. The tumors in surviving animals were both estrogen dependent. If this partial observation was evidence of the whole, it would favor a
A certain amount of ambiguity surrounds the study of the effects of exercise on mammary tumor etiology because of the broad physiological changes and adaptations that result from an extended regimen of exercise. The aim of this study was to establish the influence of a moderate, prophylactic exercise regimen on estrogen-dependent and independent rat mammary tumors and to assess this influence using parallel prophylactic protocols using tamoxifen. The hypothesis tested stated that exercise would have a broad inhibition on both estrogen-dependent and independent tumor cells; tamoxifen would have an influence on tumor growth and development with a greater effect on the estrogen-dependent tumors; and the two regimens in combination would have an additive effect on the inhibition of mammary tumor induction and growth. Though the moderate exercise regimen failed to significantly inhibit tumor growth and development when
compared with the tamoxifen protocol, the indication of a selection toward estrogen-dependent tumors does suggest that exercise does in some way influence the development of the mammary tumors. Despite the endocrine-based classification of the tumors, it is not clear whether this selection is completely or partially estrogen related.
CONCLUSIONS

The objective of this study was to establish the influence of a moderate exercise regimen on the induction and growth on the induced estrogen-dependent and independent rat mammary tumors. Examination of the hypothesis shows that the results of the experiment were different from the projections. The moderate exercise regimen did not inhibit tumor growth and appeared to select against estrogen-independent tumors. The tamoxifen protocol did significantly inhibit tumorgenesis but did not select for independent tumors. Finally, the combination prophylaxis demonstrated no additive tumor inhibition. In this study, moderate exercise in prophylaxis demonstrated no tumor inhibition and a selective pressure favoring estrogen-dependent tumors.
BIBLIOGRAPHY


Maltoni C, Pinto C, Paladini G: Project of experimental bioassays on chemopreventative gents performed at the Bologna Institute Oncology: report on tamoxifen


Sylvester PW, Aylsworth CR, Van Vugt DA, Meites J: Effects of alterations in early hormonal environment on development and hormone dependency of carcinogen-


