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## **PREVENTION OF INFLAMMATORY-RELATED LIVER DAMAGE BY TAMOXIFEN IN RATS GIVEN FISH OIL**

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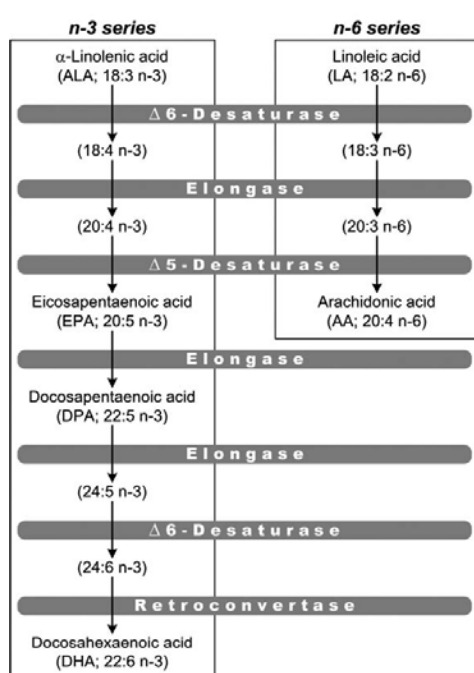
### **Abstract**

The focus of this research was to decrease inflammatory-related liver damage from tamoxifen in rats by adding fish oil to the diet. Tamoxifen causes a significant increase in inflammation of the liver. Inflammation increases with the production of prostaglandins by a metabolic pathway involving arachidonic acid. The metabolism of tamoxifen by the enzyme cytochrome P450 leads to an increase in the production of prostaglandins. The increased inflammation is proportional to lipid accumulation and ultimately lipid peroxidation in the liver. Resulting damage in humans includes hepatic steatosis (fatty liver), nonalcoholic steatohepatitis (NASH), and cirrhosis. Fish oils rich in omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), should decrease inflammation by indirectly suppressing the production of prostaglandins through enzymatic competition. Female rats were given a 7-day treatment of tamoxifen (35mg/kg) and/or fish oil (1000 mg/kg) by IP injection. Total rat weights were significantly lower in rats receiving only tamoxifen and significantly greater in rats receiving only fish oil, with no significant difference in the control rats or the rats receiving both variables. Liver weights exhibit no significant differences. Liver lipid analyses showed a significant decrease in lipid accumulation in rats that received both tamoxifen and fish oil. A significant decrease in lipid peroxidation was prominent in the rats that only received fish oil, possibly a result of its antioxidant activity. Future work may include increasing the numbers of rats and identifying proteins, estrogen metabolites, and tamoxifen metabolites, as well using a TBARS analysis for lipid peroxidation.

## Introduction

Tamoxifen is a widely used drug in the treatment of breast cancer. It is classified as a selective estrogen receptor modulator (SERM). The active metabolite, 4-hydroxytamoxifen, blocks the estrogen receptor sites to inhibit growth of tumor cells in the mammary gland. It is metabolized by a cytochrome P450 enzyme in the liver. The metabolism of drugs by cytochrome P450 results in reactive intermediates that increase liver damage [10]. It also affects the biotransformation of eicosanoids, including prostaglandins [11].

Non-alcoholic steatohepatitis (NASH) is associated with the chronic use of tamoxifen. It is caused by chronic inflammation, fibrosis, and lipid accumulation of the liver. Mechanisms of damage include mitochondrial dysfunction, the overproduction of reactive oxygen species (ROS), and lipid peroxidation [2]. Fish oils rich in omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may decrease damage by preventing inflammation. Omega-3 fatty acids and omega-6 fatty acids utilize the same metabolic enzymes, and therefore enzymatic competition occurs when omega-3 fatty acid metabolism is increased (see Figure 1). The metabolism of omega-6 fatty acids produces arachidonic acid, which results in the production of prostaglandins. Prostaglandins are classified as an eicosanoid that results in inflammation in both normal and tumor cells. Their production is inhibited by an increase in omega-3 fatty acids, found in fish oils.



**Figure 1.** Metabolic route of fatty acids [8].

Fish oils may result in an increase in lipid peroxidation products. Lipid peroxidation occurs when double bonds in lipids and fatty acids are broken down by ROS, and result in free radical formation [12]. However, fish oils increase superoxide dismutase (SOD)

and glutathione peroxidase, which protect tissues from lipid peroxidation [7]. Also, there is less lipid peroxidation if the fish oil is natural, relatively stable, and is in concentration less than 38% [15]. Fish oils are also beneficial to breast cancer patients by inhibiting eicosanoid production in cancer cells to inhibit their growth [14], and by increasing the efficacy of anticancer drugs [6, 9].

## Methods

### *Experimental Animals:*

MSU's colony of female Wistar Kyoto (WKY) rats at 10 weeks of age, weighing  $249 \pm 44$  g were given access to rat chow and water, with 2-3 rats/cage, and were acclimated for two weeks prior to experimentation. They were randomly placed into four groups of five rats each, as follows (see Table 1): **Group A** – 0.2 ml tricapyrylin vehicle and 0.3 ml saline solution; **Group B** – 0.2 ml of tamoxifen (35 mg/kg) with tricapyrylin vehicle and 0.3 ml saline solution; **Group C** – 0.3 ml fish oil from Menhaden (1000 mg/kg) and 0.2 ml tricapyrylin vehicle; and **Group D** – 0.2ml of tamoxifen (35 mg/kg) with tricapyrylin vehicle and 0.3 ml fish oil from Menhaden (1000 mg/kg). Intraperitoneal (IP) injections of all four groups were performed for seven days, accordingly, and euthanized by carbon dioxide on the eighth day.

**Table 1.** Injected variables for each group of female rats.

Group	A	B	C	D
Tricaprylin	X	X	X	X
Tamoxifen citrate (35mg/kg) (Sigma-Aldrich)		X		X
Fish Oil (1000 mg/kg) (OmegaPure, 25% EPA/DHA)			X	X
Saline Solution	X	X		

### *Lipid accumulation & cirrhosis:*

Livers were perfused with approximately 50 ml of 0.9% NaCl solution, and excised from the body. Weights and colors of rat livers were recorded for analysis of lipid accumulation and cirrhosis.

### *Liver microsomal preparation:*

Liver microsomes, drug-metabolizing enzymes including cytochrome P450, were prepared by a standard method by Omura and Sato [13]. Livers were halved (one half for later use), and weights were recorded. Excised livers were finely chopped and homogenized with 4 volumes of 1mM EDTA and 0.25 M sucrose. 10 uL of homogenate were stored in a test tube for later use. The homogenate was centrifuged at 9000 X G (Rotor JS13.1: 7576 RPM) for 10 minutes at 4° C. Precipitate was discarded and the resulting sediment microsomes were centrifuged at 112700 X G (Rotor SW27: 25000

RPM) for 60 minutes. The upper layer was removed and the pellet was resuspended in 0.1 M phosphate buffer, pH 7.0, and stored at 4° C.

#### *Cytochrome P450 Concentration:*

Cytochrome P450 (CYP) concentration was measured by standard methods by Omura and Sato [13] and Conney [4]. The microsomes were suspended in 0.1 M phosphate buffer (pH 7.0). Using a dual-beam spectrophotometer, a baseline was accomplished by running untreated microsomes in 1-cm square sample and reference cuvettes from 350 nm to 550 nm. The sample cuvette's contents were reduced in a few milligrams of solid sodium dithionate. Carbon monoxide was then bubbled into the sample cuvette. A second scan determined CYP concentration by using the  $\Delta A_{450-500}$  (peak-trough) and the extinction coefficient of  $91 \text{ mM}^{-1} \text{ cm}^{-1}$ .

#### *Protein Quantification:*

Protein quantification by Bradford assay [3] will aids in the measurement of CYP and corresponds to lipid production in the liver. A standard curve was prepared according to Table 2, using a 1.0 mg/ml bovine serum albumin (BSA) solution in distilled H<sub>2</sub>O. 5 ml of Bradford reagent were added to 100  $\mu\text{L}$  of sample, incubated at room temperature for 5 minutes, and placed in spectrophotometer at 595nm. Protein quantity was calculated using a standard curve based on the Table 2.

**Table 2.** Bradford assay recipe for preparing solutions for standard curve at A=595 nm.

$\mu\text{L dH}_2\text{O}$	$\mu\text{L BSA Solution}$	100 $\mu\text{L Final Standard}$
100	0	0
90	10	10
75	25	25
50	50	50
25	75	75
0	100	100

#### *Lipid Accumulation:*

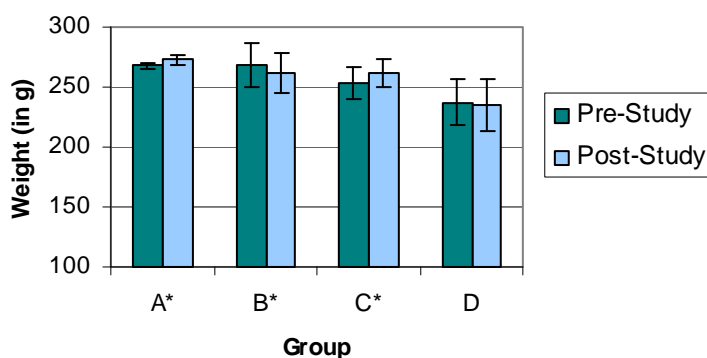
To further analyze lipid accumulation, total lipids were extracted and purified by an accepted method by Folch *et al* [5]. Liver tissues were homogenized with 2:1 chloroform-methanol mixture (v/v) and butylated hydroxytoluene (BHT) (5 mg/100ml) to a final dilution 20-fold the volume of the tissue sample. BHT was added to the sample to reduce oxidation. Homogenate was filtered and washed with 20% of the volume of water. Mixture was allowed to separate, and the upper layer was removed. Methanol was added, and the resulting solution was diluted to final volume with the 2:1 chloroform-methanol mixture. After 2 ml of water was added to the solution in a centrifuge tube, the sample was mixed and centrifuged until complete separation occurred. The resulting pellet was dried and weighed.

*Lipid Peroxidation:*

Lipid peroxidation was measured using an enzyme immunoassay kit for 9( $\pm$ )-hydroxydecadienoic acid (Oxford Biochemical Research, Inc.). Extracted lipids were diluted with 4 ml methanol, containing 5 mg BHT/100 ml and an equal volume of 15% KOH. Samples were placed in a water bath at 37° C for 30 minutes. Samples were then acidified to pH 3 using 1 N HCl, and diluted to 80 ml with pH 3 water. Water saturated ethyl acetate was added (3X sample volume), and sample was centrifuged at low speed for extraction. Organic phase and removed and transferred to clean test tube. Samples were then dried under nitrogen gas. Bio-Rad Microplate Reader, Model 550, was used to determine standard curve and amount 9( $\pm$ )-HODE present in each sample.

**Results***Rat Total Body Weight:*

Rats were weighed prior to experimental period and then again after the seventh day of injections. Pre and post weights were compared for significant differences. Group A (control) gained a significant amount of weight; Group B (tamoxifen) lost a significant amount of weight; Group C (fish oil) gained a significant amount of weight; Group D showed no significant difference (see Figure 2). Tamoxifen caused a weight loss, fish oil caused a weight gain, and fish oil restored the weight loss related to the addition of tamoxifen to the diet.



**Figure 2.** Comparison of pre-study total rat weights and post-study total rat weights. \* denotes a statistical difference.

*Liver Weight & Degree of Cirrhosis:*

After excising each liver, weights were recorded and any discoloration was noted. Comparison of liver weights between each group showed no significant differences. Also, no discoloration was noted for any of the livers.

*Total Lipids:*

Percent total lipids were calculated for each group. It was found that a significant difference existed between Group A (control) and Group D (tamoxifen/fish oil) (see Table 2).

**Table 2.** Comparison of percent total lipid between the Group A (control) and Group D (tamoxifen/fish oil), after 7-day treatment.

	<u>Group A</u>	<u>Group D</u>
Mean (percent total lipid)	5.1915	3.6053
Variance	0.7255	0.3085
P(T<=t) one-tail	0.0051	
P(T<=t) two-tail	0.0102	

*Cytochrome P450:*

Microsomes were prepared in order to quantify cytochrome P450, the enzyme used in metabolizing tamoxifen in the liver. During microsomal preparation, an error occurred that resulted in the breakdown of the products necessary for accurately quantifying cytochrome P450. Therefore, this portion of the experimental analysis was inconclusive.

*Protein Quantification (Liver and Microsomal):*

Both the liver proteins and microsomal proteins were quantified by a Bradford assay. No significant difference in liver proteins existed between the groups. However, a significant difference in microsomal proteins existed between Group B (tamoxifen) and Group D (tamoxifen/fish oil), with a p-value of 0.017.

*Lipid Peroxidation:*

Lipid peroxidation was measured by quantifying conjugated dienes (9±-HODE) using a colorimetric immunoassay kit. Resulting calculations (see Table 3) exhibited that Group C (fish oil) had significantly fewer lipid peroxidation products compared to all other groups, with a p-value of 0.029.

**Table 3.** Lipid peroxidation statistics.

Group	Mean $\pm$ SE
A	1739.70 $\pm$ 185.3
B	1715.76 $\pm$ 254.3
C	1229.50 $\pm$ 188.6
D	1312.10 $\pm$ 456.9

### Conclusions

Tamoxifen decreases body weight, while fish oil increases body weight in female rats. Fish oil restores the body weight when tamoxifen is administered to female rats, without increasing the percent of total lipids of the liver. The combination of tamoxifen and fish oil decreases lipid accumulation in the liver through mechanisms related to preventing inflammation by decreasing prostaglandin production.

Fish oil decreases lipid peroxidative damage in female rats. This may be related to an increase in SOD and glutathione peroxidase in the liver, as a result of adding fish oil to the diet.

Analyses of experimentation show that chronic affects were not seen in a 7-day treatment of tamoxifen. However, acute affects have shown to be successful in the prevention of NASH, by preventing lipid accumulation of the liver and by decreasing lipid peroxidation products.

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### **Author's Biography:**

Renae Haycraft grew up in Lewisville, Minnesota, and graduated high school at Mankato West High School in 2001. She started attending Minnesota State University, Mankato, in the fall of 2001. She is graduating this spring with a B.S. in Biology, Toxicology Option, and a B.A. in Chemistry. After graduation, she will begin employment at 3M Corporate Toxicology and Regulatory Services in St. Paul, MN.

### **Faculty Mentors' Biographies:**

Dr. Steve Mercurio received a B.A. in biochemistry (bachelor's project = circular dichroism analysis of pyruvate kinase), M.A. in biology (thesis = development of enzyme electrodes) and Ph.D. in anatomy (dissertation = development and induction of cytochrome P450 in chick embryo liver) from the University of Pennsylvania. A two-year postdoctoral position followed at the University of Minnesota's Department of Pharmacology (behavioral toxicology). Following that, he was a senior research

associate in the Department of Poultry and Avian Sciences at Cornell University (Se and antioxidant effects on damage caused by oxidant medications). He came to Minnesota State University Mankato 20 years ago to start the Toxicology program. He has had one sabbatical as an AAAS-EPA fellow in 1995. He is a member of the Society of Toxicology, Society of Environmental Toxicology and Chemistry, New York Academy of Sciences, American Association for the Advancement of Science, and American Society of Nutritional Sciences. He is married and has two adult children.

Dr. Danae R. Quirk Dorr earned her B.A. from the College of St. Scholastica - Duluth, Minn., in Biochemistry and Biology and then earned her Ph.D. in Medicinal Chemistry with a minor in Organic Chemistry from the University of Minnesota - Minneapolis, MN. Following post-doctoral studies at the University of Minnesota Cancer Center, Dr. Quirk Dorr has been an assistant professor at Minnesota State University - Mankato since 2005.