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**Thymoquinone Decreases Oxidative DNA Damage (8-OHdG) in DMBA Treated
Female Sprague Dawley Rats**

**A Thesis Presented to Eastern Washington University
Cheney, Washington**

In Partial Fulfillment of the Requirements for the Master of Science in Biology

By

Abrar A. Sindi

Spring 2013

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Thesis for Master of Science

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Abstract

Tamoxifen (TAM) is used widely for treatment and prevention of breast cancer. However, TAM has been reported to have a negative effect on glutathione (GSH) levels and 8-hydroxydeoxyguanosine (8-OHdG). Thymoquinone (TQ), the main active ingredient of *Nigella sativa*, or Black seeds, is used in the Middle East for treatment of several diseases, especially cancer. The aim of my research was to investigate the role of thymoquinone in improving 8-OHdG and GSH compared with tamoxifen in female Sprague Dawley rats treated with 7,12 dimethylbenz (a)anthracene (DMBA, a drug that induces mammary cancer). 8-OHdG is the most commonly assayed fingerprint of free radical attack on DNA and is involved in tumor progression. Five groups of 10 rats (Controls, DMBA, TAM, TQ, and TAM+TQ) were treated for 10 weeks after injection of DMBA. Tumors were found in only two DMBA treated rats. However, there was a coloration observed in the mammary glands in another four of the DMBA treated rats. There were no mammary gland changes in the other four groups. 8-OHdG in blood samples was significantly higher in the TAM group compared with the TQ ($P < 0.001$), the controls ($P = 0.008$) and the DMBA ($P = 0.041$) treated groups. TQ significantly decreased 8-OHdG by itself compared with DMBA ($P = 0.039$) and TAM, but did not significantly reduce the effect of TAM in the TAM+TQ group. On the other hand, GSH did not differ among treatments. The data for body weight gain over 12 weeks showed a significant difference among the groups ($P < 0.001$). The TAM and TAM+TQ groups had the lowest weight gain compared with the TQ, the control and the DMBA ($P < 0.01$) treated groups. I conclude that TQ could be a better anticancer drug than TAM because it decreased 8-OHdG and protected growth.

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Abbreviations

TAM = Tamoxifen

TQ = Thymoquinone

GSH = Glutathione

8-OHdG = 8-hydroxydeoxyguanosine

DMBA = 7, 12- dimethylbenz (a) anthracene

ROS = Reactive oxygen species

Introduction

- **Cancer**

Cancer is one of the most severe diseases that affect people around the world. This disease is reported to be the second most common cause of death in the United States (Soujanya et al, 2011). Cancer occurs when cell division is out of control and cells divide quickly. This quick division creates a tumor which blocks or invades normal tissues.

According to American Cancer Society one in eight women will develop invasive breast cancer. Breast cancer occurs when cells grow and multiply in uncontrolled way in breast tissue (Figure 1).

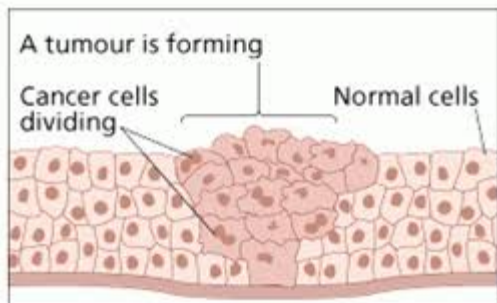


Figure 1: Loss of normal growth control in cancer cells

Tamoxifen (TAM) is a drug used currently for treatment and prevention of breast cancer. Herbs also sometimes are used for prevention and as a cure of cancer.

Thymoquinone (TQ), the main active ingredient of *Nigella sativa*, or Black seeds, is used in the Middle East for treatment of several diseases, especially cancer. The purpose of my study was to compare TAM and TQ.

- **Tamoxifen (TAM)**

In the early 1970s, the first study of a new antiestrogen called tamoxifen (TAM, ICI 46474) in breast cancer patients was published (Cole et al., 1971). In 1990, TAM was approved by the United States Food and Drug Administration (FDA). TAM citrate, a derivative of triphenylethylene (Curtis, 2001), is now frequently prescribed, and compelling data have demonstrated a significant overall survival benefit with the administration of this drug in breast cancer patients with endocrine responsive disease (EBCTCG, 1998; Clarke, 2003). Several TAM derivatives are now available, including toremifene (chloro-tamoxifen) and droloxifene (3- hydroxytamoxifen). Not surprisingly, both drugs are essentially equivalent to TAM in terms of their antitumor activities and

toxicities (Roos et al., 1983; Pyrhonen et al., 1999), so neither is widely used in clinical practice.

TAM is the most widely prescribed Selective Estrogen Receptor Modulator. SERMs, also called antiestrogens, compete with estrogens for binding to the Estrogen Receptor (ER). ERs bind estrogen as well as other agonists or antagonists. The first form of ER identified was ER α . Its activity is influenced by coactivator and corepressor proteins that can either positively or negatively modulate ER α -mediated transcriptional activity (Dobrzycka et al., 2003). The more recently discovered form is ER β which is encoded by a different gene. Target cells for estrogen action may contain varying concentrations of homodimers of one or both ERs (Gennari et al., 2007). ER α and β are different in their structure and location. For example, ER α is documented to be found in breast cancer cells (Yaghmaie et al., 2005), while ER β is found in the heart and kidney (Babiker et al., 2002). Thus, different ERs have different responses to a SERM.

The clinical response to antiestrogens may be affected by exogenous estrogenic exposures. These exposures include estrogenic hormone replacement therapies, as well as dietary or environmental exposures that directly or indirectly increase a tissue's estrogenic environment (Clarke et al., 2003). However, SERMs were given their name because of their unique pharmacologic properties (Gennari et al., 2007). SERMs can treat and prevent breast cancer by interacting with intracellular estrogen receptors in target organs as estrogen receptor agonists and antagonists. SERMs can achieve the beneficial effects of estrogens in nonmammary tissues such as bone, but have an estrogen

antagonistic effect in mammary tissue (Curtis, 2001). SERMs act by binding to the ER, inducing a characteristic conformational change that enables its interaction with specific DNA sequences in the regulatory regions of target gene promoters (Haynes and Dowsett, 1999). SERMs induce a conformational change within the ER that is different from that caused by estradiol, the main naturally occurring estrogen, and this leads to differential recognition of the specific ligand-receptor complexes by the cellular transcriptional machinery (McDonnell et al., 1995; Beekman et al., 1993).

The Early Breast Cancer Trialists' Collaborative Group tested TAM on more than 37,000 women in 55 separate randomized trials prior to 1990. The group found that TAM significantly reduced cancer recurrence in women with estrogen receptor positive tumors after 5 years of treatment (Early Breast Cancer Trialists' Collaborative Group, 1998). According to the findings from the international ATLAS (Adjuvant Tamoxifen, Longer Against Shorter) study, ten years of treatment with tamoxifen was significantly better than the standard 5 years in reducing the risk for breast cancer recurrence (Mulcahy, 2012). American Cancer Society reported that 75% of all breast cancers are ER positive (ER+). They grow in response to the hormone estrogen (Early Breast Cancer Trialists' Collaborative Group, 1998).

In a placebo-controlled trial performed in the US, the National Surgical Adjuvant Breast and Bowel Project (NSABP)-P1 trial tested 13,388 women at high risk for developing breast cancer who were treated with TAM (20 mg/day) for a 55 month follow-up trial. In this trial they found that TAM decreased the risk of invasive and non-

invasive breast cancers in all age groups by nearly 50% and decreased the incidence of ER positive breast cancer risk by 69% (Fisher et al., 1998; Curtis, 2001).

When compared with cytotoxic chemotherapy, antiestrogens have a low incidence of serious side effects and are associated with mostly minor toxicities. Three large, randomized, chemoprevention studies with TAM have been conducted: the NSABP P-1 trial (n=13,388 participants) (Fisher et al., 1998), the Royal Marsden Trial (n=2471 participants) (Powles et al., 1998), and the Italian Chemoprevention Trial (n=5408 participants) (Veronesi et al., 1998). The Italian Trial reported that TAM decreased the breast cancer risk by 82% among women at high risk for ER+ breast cancer (Veronesi et al., 2003). In the NSABP trial, a decreased breast tumor incidence was seen only for ER+ tumors (Fisher et al., 1998). The P-1 trial reported a significant decrease in the incidence of both noninvasive (50%) and invasive (49%) breast cancers (Clarke et al., 2003). From 1990 to 2000 breast cancer mortality in the United States declined about 24% due to the increased use of both mammographic screening and adjuvant therapy including chemotherapy and antiestrogens (Ariazi et al., 2006).

Some dietary components can modify the ability of TAM to inhibit the growth of ER+ and perhaps also ER- breast cancer cells. Dietary components could either potentiate or inhibit TAM actions. Ju et al. (2002) and Depypere et al. (2000) showed that genistein, which found in soybeans and other plants, or tangeretin, which present in citrus fruits, prevent TAM from inhibiting growth of malignant breast cells. In contrast, a few studies have examined the positive effect of nutrition on TAM ability to inhibit the growth of

breast cancer cells (Clarke et al., 2003). For example, the administration of *Holooptelea integrifolia* plant compared with TAM gave a significant reduction of the tumor in six female Sprague-Dawley rats after 120 days of treatments (Soujanya et al., 2011).

- ***Nigella Sativa* and Thymoquinone (TQ)**

Alternative medicine in Saudi Arabia is very popular. For Islamic culture there are many things that are described by god or by his prophets. *Nigella Sativa* is one of the most popular seeds mentioned by Prophet Mohammad. He said that these black seeds are a cure of everything except death. Some scientists have tested the effect of these seeds on several organs and diseases because of this historical and religious background.

N. sativa is an annual capsulated plant used as a spice. It is a dicotyledon of the Ranunculaceae family. It is usually grown in Europe, Middle East, and Western Asia (Kanter et al., 2003). It is frequently used in folk medicine in the Middle East and Asia for the promotion of good health and treatment of many ailments including fever, common cold, headache, asthma, rheumatic diseases, various microbial infections, and to expel worms (Salama, 2010). *N. sativa* contains 0.40–1.50% of volatile oil (essential oil contains the volatile aroma component). The volatile oil has been shown to contain 18.4–24% thymoquinone and 46% other monoterpenes such as p-cymene, and a-pinene (El-Tahir et al., 1993), and to have insecticide, bronchodilator, immunomodulative (El-Kadi and Kandil, 1987), antibacterial (Hanafy and Hatem, 1991), hypotensive (Zaoui et al., 2000), choleric, antitumoral (Salomi et al., 1992), antifungal, antihelminthic and antiasthmatic (El-Tahir et al., 1993; Kanter et al., 2003) properties.

The fixed oil (nonvolatile oil) of *N. sativa* is rich in unsaturated fatty acids while the essential oil contains thymoquinone and carvacrol, which are antioxidants. Its seed contains proteins, alkaloids (nigellines and nigellidine), and saponins (α -hederin) in substantial amounts. A review by Butt and Sultan (2010) described the effects of these seeds on the improvement of oxidative stress through free radical scavenging activity, the creation of apoptosis of cancerous cells, a decrease of blood glucose, and a protection of complications from diabetes. *N. sativa* regulates hematological and serological parameters and can be effective in dyslipidemia and respiratory disorders (Salama, 2010). Most important to my study, thymoquinone is the component which had been shown to treating breast cancer (Woo et al., 2011)

- **Reactive oxygen species and free radicals**

Reactive oxygen species (ROS) are products of a normal cellular metabolism and play roles in stimulation of signaling pathways in plant and animal cells in response to changes of intra- and extracellular environmental conditions (Reuter et al., 2010). Some ROS also contribute to pathophysiological conditions including mutagenesis and carcinogenesis.

Our bodies constantly use oxygen for aerobic synthesis of ATP. As a consequence of oxidative phosphorylation, highly reactive molecules, called free radicals, escape from mitochondria (Worden, 2011). Unmanaged oxidation results in free radicals such as ROS and reactive nitrogen species (NOS) that cause oxidative stress (Opara, 2004; Vina et al.,

2006). Free radicals contribute importantly to tumor production by direct chemical reaction or alteration of cellular metabolic processes (Soujanya et al., 2011).

These free radicals have been implicated in mediating various pathological processes such as cancer, inflammatory diseases, diabetes, and atherosclerosis (Vina et al., 2006; Wilson and Demmig-Adams, 2007).

In addition, ROS cause inflammation and carcinogenesis either directly by oxidation, nitration, or halogenation of nuclear DNA, RNA, or lipids, or indirectly by the signaling pathways activated by ROS. An example of ROS is the superoxide anion (O_2^-). Manganese-superoxide dismutase (Mn-SOD) is the main endogenous enzyme for protection against O_2^- by converting it into H_2O_2 and water (Karihtala and Soini, 2007). H_2O_2 is another example of ROS, and may be formed either by dismutation from superoxide anion or spontaneously in peroxisomes from molecular oxygen (Mates and Sanchez-Jimenez, 2000; Valko et al., 2004). H_2O_2 plays an important role in generating cancer because it is capable of diffusing out of the mitochondria and across cell membranes to create oxidative damage within and outside the cell (Mates and Sanchez-Jimenez, 2000; Ray G, Husain, 2002). H_2O_2 stress led to time-dependent increases in intracellular oxidants, mitochondrial membrane polarization, cytochrome c release, lysosomal rupture, and cell apoptosis (Yin et al., 2005). The hydroxyl radical ($\cdot OH$) usually resolves the damaging effect by ROS in the cell. It has a very unstable electron structure and it's unable to diffuse more than one or two molecular diameters before it reacts with any cellular component (Reuter et al., 2010).

- **Antioxidant and anticancer effects of *Nigella Sativa* and Thymoquinone**

Free radicals are normally kept in check by the action of free radical scavengers that occur naturally in the body. These scavengers neutralize the free radicals. Certain enzymes serve this vital function, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These antioxidants also may protect against the toxicity of ROS by the prevention of their formation (Soujanya et al., 2011). The administration of antioxidant vitamins and trace elements is important for protecting the body from oxidative stress and free radicals.

N. sativa seeds and extracts have been reported to be important in the treatment of several diseases. They inhibit lipid peroxidation and prevent the formation of carbon tetrachloride (CCI-4, which induces reactive free radicals which initiate cell damage) in rats (Al-Ghamdi., 2003). Kanter et al. (2008) mentioned that *N. sativa* oil improves the antioxidant defense mechanism in CCI-4 treated rats. In another study *N. sativa* provided a protective effect against oxidative damage in isolated rat hepatocytes (Daba and Abdel-Rahmen, 1998).

Evidence supports an effect of *N. sativa* on immunity and inflammation. Nitric oxide (NO) is an important factor in physiological processes and in disease states characterized by inflammation. *N. sativa* extract caused a decreased NO production by murine macrophages that also validated the traditional use of the *N. sativa* seeds for the treatment of rheumatism (Mahmood et al., 2003). El-Kadi and Kandil (1986) studied the effect of *N. sativa* on immunity after administering 1 g of these seeds twice a day to human

subjects. Their results suggested that *N. sativa* improved immune functions by enhancing helper T-cell (T4) to suppressor T-cell (T8) ratio and improving natural killer cell activity.

An important effect of *N. sativa* is its anticancer effect. Its components are effective in reducing cancer cell growth by the induction of apoptosis by tumor necrosis factor alpha (TNF- α) (Butt and Sultan, 2010). The administration of *N. sativa*, melatonin and retinoic acid reduced the carcinogenic effects of 7, 12 di-methylbenz(a)anthracene (DMBA), a drug used to induce mammary cancer experimentally. The frequency of mammary cancer was high in the DMBA group (60%), followed by the treated groups which got the drugs after administering DMBA (56%) and finally the prophylactic groups which got the drugs before administering DMBA (33%) suggesting a protective role of *N. sativa* against mammary cancer (El-Aziz et al., 2005).

Moreover, thymoquinone (TQ) has been reported to be effective in treating cancer. Woo et al. (2011) investigated the effect of TQ on breast cancer cells *in vitro*. Three different breast cancer cell lines (MCF-7, MDA-MB-231 and BT-474), each with distinct characteristics, were used. MCF-7 and BT-474 are estrogen receptor (ER)-positive, and MDA-MB-231 is ER-negative (Lacroix et al., 2004). The anti-proliferative effect of TQ was determined by the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT solution was added to cancer cells after treatment with TQ and left for 4 h. Cells were measured for their ability to reduce the yellow dye, MTT, to a purple. The effect of TQ on the cell cycle was determined using flow cytometry analysis.

The migration of cells was investigated using a 'wound-healing' assay. The protein expression was investigated using immunoblotting. They found that TQ suppressed the growth of all three breast cancer cell lines in a dose- and time-dependent manner. TQ induced apoptosis in MCF-7 cells through the activation of caspases and increased Bax/Bcl-2 ratio. The increase in total Bax/Bcl-2 ratio suggested that this might be one of the mechanisms of TQ-induced apoptosis in MCF-7 cells. The migration of the cancer cells was reduced with TQ-enriched medium, in a dose dependent manner. They also combined TQ with doxorubicin and 5-fluorouracil (anticancer drugs) and found an increased cytotoxicity indicating apoptotic induction.

Another study by Effenberger-Neidnicht and Schobert (2011) investigated the anticancer affect of TQ and doxorubicin in human cancer cells. Doxorubicin (drug used for cancer), TQ, and mixtures of both were tested for cytotoxicity on human cells of HL-60 leukemia, 518A2 melanoma, HT-29 colon, KB-V1 cervix and MCF-7 breast carcinomas. Apoptosis induction was analyzed by DNA fragmentation, activity studies of the caspases-3, -8 and -9, determination of changes in the mitochondrial membrane potential and in the ratio of the mRNA expressions of pro- and anti-apoptotic proteins bax and bcl-2. The generation of ROS was assessed by the nitro blue tetrazolium (NBT) assay. Their results showed that TQ improved the anticancer properties of doxorubicin in a cell line-specific manner. They found a significant increase of the growth inhibition by doxorubicin in combination with TQ. TQ was found to be a booster for the anti-cancer effect of doxorubicin in certain cancer cell lines. Effenberger-Neidnicht and Schobert

suggested that distinct improvements in efficacy, selectivity, and even breaches of multi-drug resistance were observed for equimolar mixtures of doxorubicin and TQ.

- **Oxidative DNA Damage and 8-OHdG**

Antioxidant defense systems cannot provide complete protection from noxious effects of ROS. These effects include oxidative DNA damage, which animal and *in vitro* experiments have suggested are important in carcinogenesis (Loft and Poulsen, 1996). More than 100 modifications of DNA from ROS have been described including different modifications of purines, pyrimidines, single-strand breaks and double-strand break (Poulsen et al., 1998).

The $\cdot\text{OH}$ -derived DNA damage includes the generation of 8-hydroxyguanosine (8-OHG), the hydrolysis product of which is 8-hydroxydeoxyguanosine (8-OHdG). 8-OHdG is the most commonly assayed fingerprint of free radical attack towards DNA (Marnett, 2002; Wiseman and Halliwell, 1996).

8-OHdG is involved in tumor progression. 8-OHdG has been reported to be enhanced 8- to 17-fold in breast primary tumors compared with normal breast tissue (Malins and Haimanot 1991; Musarrat et al., 1996; Reuter et al., 2010). An elevated level of urinary 8-OHdG has been detected in patients with various cancers (Wu et al., 2004).

- **Oxidative DNA Damage and Tamoxifen**

A study by Bhimani et al. (1993) reported that TAM (2.5-20 μM per cell) decreased the 12-O-Tetradecanoylphorbol-13-acetate (TPA)-mediated oxidative stress effect on 5-hydroxymethyl-2'-deoxyuridine (HMdU) and 8-OHdG formation 2-29 times in HeLa (cervical cancer) cells. Maximum inhibition occurred at 20 μM TAM, which caused an approximately 95% reduction in HMdU and 8-OHdG. They were investigating TPA effect on oxidative stress in HeLa cells and the inhibition effect by TAM using ^3H -postlabeling of 8-OHdG. TPA treatment (10 fmol/cell) caused about 5-10-fold increase in 8-OHdG and HMdU (10 nmol TPA/ml), while TAM caused reduction of both. Their finding suggested that TAM is a chemopreventive agent, which at very low doses decreases the tumor promoter-mediated oxidative processes.

However, their study did not test the effect of TAM on DNA damage of normal cells. TAM treatment can lead to DNA damage in normal cells, but the mechanism of this process is not fully understood. Wozniak et al. (2007) compared the DNA-damaging potential of TAM in MCF-7 breast cancer cells and normal human peripheral blood lymphocytes by using the comet assay. For evaluating the involvement of free radicals in the genotoxicity of TAM they pretreated the cells with nitron spin traps: DMPO and POBN. Vitamin C, amifostine and genistein in breast cells were used to evaluate the contribution of free radicals. TAM damaged DNA in normal and cancer cells, causing DNA strand breaks. TAM at 5 and 10 μM induced DNA double strand breaks in normal lymphocytes and at 10 μM in MCF-7 cells. In both types of cells TAM induced oxidized purines and pyrimidines. Their results suggested that TAM can be genotoxic for normal

and cancer cells by free radical generation. TAM also had a higher genotoxic potential for normal cells, which could be the result of incomplete repair of DNA double strand breaks. They mentioned that free radical scavengers can modulate TAM-induced DNA damage interfering with its antitumor activity in cancer cells.

- **Oxidative DNA Damage and Thymoquinone**

The concerns about cancer drugs and DNA damage made scientists dedicate their efforts to finding natural treatments such as *N. sativa*. Koka et al. (2010) investigated the mechanism of action of TQ in androgen receptor (AR)-independent (C4-2B) and AR naive (PC-3) prostate cancer cells. They exposed cells for 24–48 h to TQ (25–150 μ mol/L) and observed inhibition of the growth of both types of cancer cells. TQ also up-regulated the expression of growth arrest and DNA damage inducible gene (GADD45a) and apoptosis-inducing factor-1.

Another study by Rastogi et al. (2010) investigated the protective effect of *N. sativa* against radiation-induced oxidative damage. Single cell gel electrophoresis assay and micronuclei formations were used to determine DNA damage. Biochemical methods were used to determine the alterations in lipid peroxidation and antioxidant enzymes. Using mouse splenic lymphocytes treated with an ethanolic extract of *N. sativa* 1 h prior to irradiation caused a significant prevention of the formation of lipid peroxides and intracellular ROS. Moreover, they found prevention of DNA damage which was measured by the bone marrow micronuclei assay.

As mentioned previously, Woo et al. (2011) investigated the effect of thymoquinone (TQ) on breast cancer cells. They found that TQ suppressed the growth of all three breast cancer cell lines in a dose- and time-dependent manner. TQ induced apoptosis in MCF-7 cells. It is also reduced the migration of the cancer cells. Moreover, when TQ was combined with doxorubicin and 5-fluorouracil (anticancer drugs) there was an increase in cytotoxicity to the cancer cells. They also reported for the first time that TQ was able to increase peroxisome proliferator-activated receptor gamma (PPAR- γ) activity which down-regulates the expression of the genes for Bcl-2, Bcl-xL. PPAR- γ activation leads to growth inhibition, apoptosis and differentiation of tumor cells. More importantly, the increase in PPAR- γ activity was prevented in the presence of PPAR- γ specific inhibitor and PPAR- γ dominant negative plasmid, suggesting that TQ may act as a ligand of PPAR- γ .

A study by Gurung et al. (2010) was performed to investigate the cytotoxic effect of TQ in brain tumor and normal cells. They demonstrated that glioblastoma (brain cancer) cells were more sensitive to TQ induced antiproliferative effects than were normal cells. TQ caused DNA damage, cell cycle arrest and apoptosis in the glioblastoma cells. The single-cell gel electrophoresis (comet assay) under alkaline conditions was used to determine the DNA damage.

- **Glutathione**

Glutathione (GSH) is a tripeptide and the major endogenous antioxidant produced by animal and plant cells. It helps to protect cells from ROS, free radicals, and peroxides. GSH has a role in regulating cell apoptosis. GSH is emerging as a regulator of the expression of proteins (or molecules) involved in apoptosis or inflammation. In the cytoplasm, overproduction of ROS and the resulting decrease in GSH activates various transcription factors (like p38 protein) which migrate into the nucleus to activate gene transcription. Therefore, glutathione plays a primary role in maintaining a normal redox status and in regulating transcription of genes (Pincemail et al., 2001).

GSH is present inside cells mainly in the reduced form (90–95% of the total glutathione). Oxidation of glutathione leads to the formation of glutathione disulfide (GSSG). The glutathione transferases (GSTs) are one of the most important families of detoxifying enzymes in nature. The classic activity of the GSTs is conjugation of compounds with electrophilic centers to GSH (Oakley, 2011)

In terms of cancer prevention, GSH can inhibit cancer development through phase II metabolism and subsequent export of toxic chemicals from the cell. (Konishi et al., 1997; Alliangana, 1996; Reuter et al., 2010). The pi-class glutathione S-transferase (GSTP1) activity, for example, protects cells from carcinogens in the human prostate. Kanwal et al. (2012) studied the protective effect against oxidative DNA damage and stress by GSTP1 in human prostate. They found high levels of 8-OHdG in adenocarcinomas, compared to benign counterparts, which positively correlated ($r = 0.2$) with loss of GSTP1 activity.

Suppressing GSTP1 using an siRNA approach in normal prostate cells caused elevated intracellular production of ROS. In contrast, induction of GSTP1 activity lowered endogenous ROS levels in cells and significantly reduced 8-OHdG levels compared to the controls. This study suggested a negative correlation between glutathione S-transferase and 8-OHdG levels.

TAM was observed to reduce GSH production. A study using TAM by Bruning et al. (2010) observed a significant reduction of the GSH level by 7% for MCF7 cells treated with 6 µg /ml TAM. In contrast with using a different drug (nelfinavir) they observed more reduction in the intracellular GSH than TAM in concentration of 6 µg /ml. The combination of nelfinavir and TAM did not reduce the GSH levels in breast cancer cells in a significant manner like nelfinavir by itself. However, the intracellular GSH levels might vary not only because of external drug applications, but also due to differences in cell growth, nutrient concentrations, and the redox state within the cells or of the surrounding medium. These errors could be avoided by using animal models and measuring the overall levels from the body. Moreover, oxidative DNA damage was not related to GSH. More investigation is needed.

In contrast, TQ enhances GSH formation. Quinones, naturally found in the human body and environment, are highly reactive molecules that can be metabolized to free radicals and cause oxidative damage. However, quinone reductase (QR) has been found to protect against quinone toxicity (Cheng, 2006). TQ induces QR and GSH transferase in mouse liver. Nagi and Almakki (2009) found a possible role in protection against

chemical carcinogenesis and toxicity by TQ. Protection could be mediated by induction of detoxifying enzymes, including quinone reductase and glutathione transferase. TQ was administered to mice (1, 2 and 4 mg/kg/day) for five days to measure quinone reductase and glutathione transferase in livers. TQ uptake increased the activities of quinone reductase (147, 196 and 197% of control, respectively) and glutathione transferase (125, 152 and 154 % of control, respectively). This result makes TQ appear to be a promising preventive agent against chemical carcinogenesis and toxicity.

A recent study by Harzallah et al. (2012) reported a significant increase in the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) enzyme levels by 50.23 %, 12.55 %, and 352.9 %, compared to controls in rats with colon cancer treated with TQ. They used 25 rats divided into 5 per group and treated weekly. The five groups were: 1- saline, 2-TQ, 3-DMH procarcinogen, 4- pre-treatment (TQ + DMH) (10 weeks), and 5- post-treatment DMH cancer inducer drug (10 weeks) + TQ (10 weeks) treated rats. DMH dose was 20mg/kg bw and TQ dose was 5mg/kg bw.

- **Introduction summary**

In summary, numerous *in vitro* and *in vivo* studies have provided evidence that TQ could prevent carcinogenesis and inhibit tumorigenesis through different molecular mechanisms. They determined the antioxidant enzyme activities by using assay kits for catalase activity, superoxide dismutase activity and glutathione peroxidase activity (Woo et al., 2012; Harzallah et al., 2012). On the other hand, TAM is reported to be effective

in the prevention and protection against cancer, but it has a negative effect on GSH levels and 8-OHdG. TAM was reported to damage DNA in normal and cancer cells (Wozniak et al., 2007).

Some dietary components however can modify the activity of TAM. The concerns about cancer drugs and DNA damage made scientists dedicate their efforts to find natural treatments like *N. sativa*. TQ was reported to be effective as an anticancer treatment (Woo et al., 2011). TQ was reported to prevent DNA damage. TQ also was able to increase PPAR- γ activity which down-regulates the expression of some genes (Woo et al., 2011). In addition, TQ was found to induce QR and GSH transferase activity (Rastogi et al., 2010).

- **Mammary cancer induction in rats**

Scientists have discovered many specific drugs that induce cancer in rats. Mammary glands of several rat strains, mainly Sprague-Dawley, are susceptible to transformation induced by chemical carcinogens, and the two most widely used active chemical inducers of mammary carcinogenesis are 7,12 dimethylbenz (a)anthracene (DMBA) and N-methylnitrosurea (Barros et al., 2004). DMBA produces rat mammary cancer and has been widely used in cancer studies since it was first reported by Huggins et al. (1961) (Motoyama et al., 2005). One dose of DMBA by oral gavage leads to up-regulation of the cellular cytosolic receptor for DMBA, the aryl hydrocarbon receptor (AhR) (Trombino et al., 2000). This activated AhR/ARNT complex then binds to specific DNA recognition

sites upstream of AhR responsive genes and causes gene transcription which in the end forms DNA adducts (Denison and Nagy, 2003).

Hypotheses

My first hypothesis was that TQ would decrease 8-OHdG in rats' plasma more than TAM. I also predicted that the combination of TAM and TQ would reduce 8-OHdG more than TAM by itself. 8-OHdG is the most commonly assayed fingerprint of free radical attack on DNA (Marnett, 2002; Wiseman and Halliwell, 1996). 8-OHdG is also involved in tumor progression.

My second hypothesis was that that TQ would increase the production of GSH more than TAM in Sprague-Dawley rats. I also predicted that the combination of TAM and TQ would have the best result in enhancing GSH production. GSH plays an important role in protecting cells from ROS and 8-OHdG, both of which are involved in cancer. It is now well understood that ROS can damage DNA. GSH on the other hand can protect against this type of damage (Kanwal et al., 2012).

Methods

- **Animals**

Fifty female Sprague-Dawley rats were obtained from the EWU vivarium. Rats were born August 8-15, 2012, were between 179-261 g body weights.

The number of rats was 10 per group in five groups. This number was determined by a power analysis (Quinn & Keough, 2002). Mishra et al. (2012) used six rats per group and treated rats with TAM. The power of their experiment was 0.05 which is so low due to the sample size. In contrast, Kanter et al. (2003) used 15 rats per group and treated them with TQ. The power of their experiment was 1. Another study by Singh et al. (2011) used at least 8-10 rats samples to find the effect of TAM on 8-OHdG. Their power was 0.97. These findings suggested that six rats are not enough and 15 are too many, while 10 would be sufficient.

The rats were housed in pairs in the EWU vivarium and allowed free access to water and Teklad 8640 Standard Rodent Diet. The room temperature was 26 ± 1 °C, humidity was 25-30% and photoperiod was a 12-hour light/12-hour dark cycle. Body weight was recorded weekly. Cages were cleaned weekly and changed every 4 weeks. I got approval from the EWU Institutional Animal Care and Use Committee before I began rat treatments.

- **Treatments**

There were five different groups (n=10 per group):

1. Controls: received tap water only, daily for 10 wk.
2. DMBA group: received one dose of DMBA, and tap water daily for 10 wk.
3. TAM group: received one dose of DMBA, and TAM daily for 10 wk.
4. TQ group: received one dose of DMBA, and TQ daily for 10 wk.
5. TAM + TQ group: received one dose of DMBA, and TAM + TQ daily for 10 wk.

A first dose of DMBA was given by oral gavage to the following groups: DMBA, TAM, TQ, TQ+TAM. After three weeks, the other drugs were administered by oral gavage 7 days per week for 10 weeks. A second dose of DMBA was administered to the same groups in the 4th week because of an insufficient first dose due to incorrect measurements with the oral gavage needle.

For oral gavage I extended the rat's head back using an index finger on top of the head to raise the head so the esophagus was in a straight line. Next, I inserted the gavage needle into the rat's mouth. Once the gavage needle was properly placed with the end of the gavage needle in the rat's stomach, I slowly administered the dose. After administration, I slowly removed the gavage needle from esophagus.

- **Drugs and drug administration**

DMBA was purchased from Sigma Aldrich and was mixed with corn oil with a shaker. Corn oil was purchased at a local grocery store. A DMBA dose of 20 mg /kg (Motoyama et al., 2008) was administered. The total volume of DMBA needed for all of the 40 rats was determined. For the average body weight of 250 g, the total volume of DMBA was 200 mg. Therefore, I prepared a solution containing 200 mg of DMBA mixed with 10 ml of corn oil. The dose in ml per rat was calculated as following: the rat weight in g/ 1000. For example if the rat weight was 250 g the dose was 0.25 ml of the solution.

Because DMBA induces cancer and contains a component that has been reported to be probably carcinogenic for humans, I protected myself as follows:

1. DMBA was locked in safety box.
2. I covered all the surfaces such as walls, desks, the fume hood and door handles with plastic.
3. I put on disposable lab coats, long double gloves, face shield and mask.
4. Rats were given DMBA under the fume hood to minimize contamination.
5. The insides of rats' cages were covered with absorbent sheets so that vomiting could be observed and contained.
6. Each rat was placed under the fume hood and given the dose and observed for 5 minutes in its cage.
7. After finishing all the rats I removed the absorbent sheets from their cages and cleaned the outside surface of the cages with 70% alcohol.
8. After that I put all of the sheets and covers with gavage needles, lab coats, gloves and DMBA container into hazard disposal bags and cleaned the room with soap and 70% alcohol.
9. After one week the rats' bedding was sent to hazard disposal.
10. After two weeks the rats' cages were cleaned and washed twice in EWU Vivarium's cage washer followed by two empty cycles of the cage washer to minimize contamination of the cage washer.
11. I washed the rats with clean water and placed them under a heating lamp until they dried.
12. Rats were then placed into their clean cages.

TAM was purchased from Cayman Chemical. The TAM dose was 10 mg/kg /day dissolved in water (Soujanya et al., 2011). The total volume of TAM needed for all 20 rats was determined. For the average body weight of 250 g the total volume of TAM was 50 mg. I prepared a solution containing 50 mg of TAM dissolved in 5 ml water daily. The dose in ml per rat was calculated as following: the rat weight in g/ 1000.

TQ was purchased from Sigma Aldrich. The dose of TQ was 20 mg/kg/day dissolved in corn oil (Nagi and Almakki, 2009). The total volume of TQ needed for all 20 rats was determined. For the average body weight of 250 g the total volume of TQ was 100 mg. I prepared a solution containing 100 mg of TQ mixed with 10 ml of corn oil daily. The dose in ml per rat was calculated as following: the rat weight in g/ 1000.

The TAM+TQ group received the same doses of TAM and TQ. The solutions were prepared as described previously. The dose was administered in the same oral gavage at the same time. The maximum volume for 250 g rat was 0.25 ml of TAM solution and 0.25 of TQ solution.

- **Blood Samples**

After 10 weeks of treatment blood was collected by cardiac puncture. Before cardiac puncture, each rat was anesthetized with isoflurane, using Dr. Carlberg's chamber method. The rat was removed from the chamber when it was unconscious, and its nose was placed in a cone containing isoflurane-soaked cotton. After cardiac puncture the rat was placed in another chamber, with a higher level of isoflurane, for euthanasia.

The following procedure was described by Beeton et al. (2007) and was modified according to Dr. Carlberg's method:

1. 5 ml of distilled water were added to one heparin tablet (Sigma Aldrich H3393).
2. 21G X 1.5 inch needles with 3 cc syringes were coated with heparin to prevent blood clotting during cardiac puncture.
3. Two chambers were prepared for isoflurane by adding layers of paper towels in their bottoms.
4. The first chamber contained 6 ml of isoflurane, while the second one contained 15 ml of isoflurane.
5. Nose cotton was prepared with 3 ml of isoflurane in case rats awakened.
6. A rat was anesthetized in the first chamber.
7. The rat was moved around inside the chamber to determine if it was anesthetized.
8. The rat was placed on its back, facing away from me.
9. My left index finger was placed at the level of the lowest ribs, without applying any pressure. The heart was located ~ 1 cm above this point, slightly to the rat's left side. After feeling the heart the needle was inserted below the xiphoid and through the diaphragm. Blood was drawn by inserting the needle into a ventricle. Approximately 3 ml of blood was drawn from each rat.
10. After cardiac puncture the rat was placed into the second chamber, with the higher level of isoflurane, for euthanasia.

11. Needles were placed in the sharps container and blood samples were moved to the cold centrifuge at 4 °C.

Blood samples were centrifuged at 600 x g, 4 °C for 10 minutes to separate red blood cells from the plasma. Red blood cells were used for the GSH assay, and plasma was used for the 8-OHdG measurement. I separated the plasma from cells and divided the plasma into three aliquots, one for the 8-OHdG test and the other two for Nadiyah Al-Otaibi's studies. The red blood cells were saved in different aliquots for the GSH test. All aliquots were labeled and frozen at -80 °C.

- **Oxidative DNA Damage ELISA Assay (8-OHdG Quantitation)**

The oxidative DNA damage ELISA assay kit was obtained from Cell Biolabs, Inc. I used the protocol from the product manual. In summary, this kit is an enzyme immunoassay made for detection and quantitation of 8-OHdG in plasma, or other samples. I used plasma to detect 8-OHdG quantity. The quantity of 8-OHdG in a plasma sample is found by comparing its absorbance with a 8-OHdG standard curve.

Before starting the assay I did a practice test and several evaluations of extra plasma from three rats to determine the best dilution for the standard curve. I figured out that a 20 fold dilution was the best to use. In addition, I needed to separate the rats into two plates, each containing half of the rats because one plate was not enough for all of the samples in duplicates. Therefore, I did the test twice.

The 8-OHdG assay included the following steps:

1. Plasma was diluted 20 fold by adding 10 μ l of thawed plasma to 190 μ l Assay Diluent.
2. For coating the plate: 8-OHdG Conjugate was diluted 1 mg/mL to 1 μ g/mL of 1X PBS. Then 100 μ L of the 1 μ g/mL 8-OHdG Conjugate was added to each well and incubated overnight at 4 °C.
3. On the following day a spectrophotometer was prepared using 450 nm.
4. For washing the plate: Wash Buffer (100 ml) was Diluted by adding 900 ml of distilled water.
5. The 8-OHdG coating solution was removed and washed once with distilled water and the plate was blotted on paper towels to remove excess fluid.
6. 200 μ L of Assay Diluent was added to each well and kept for 1 hr at room temperature.
7. Then the plate was transferred to 4°C or Assay Diluent was removed immediately before the next step.
8. A 8-OHdG standard curve was prepared using the product manual (Table 1).
9. Rats' plasma and the standard curve solutions could not all fit into one 96 well plate. Therefore, they were divided into two separate plates, each containing five rats' plasma from each group and their standard curve.
10. 50 μ l of diluted plasma or 8-OHdG standard were added into the wells in duplicates and incubated at room temperature for 10 minutes on an orbital shaker.
11. Anti-8-OHdG Antibody was diluted 1:500 with Assay Diluent.
12. Rats' plasma was removed from the freezer 30 minutes before the assay started.

13. 50 μ L of the diluted anti-8-OHdG antibody was added to each well and incubated at room temperature for 1 hour on an orbital shaker.
14. Secondary Antibody was diluted 1:1000 with Assay Diluent.
15. Micro-well strips were washed 3 times with 250 μ l 1X Wash Buffer per well with aspiration.
16. 100 μ L of the diluted Secondary Antibody-Enzyme Conjugate was added to all wells and incubated at room temperature for 1 hour on an orbital shaker.
17. The Substrate Solution was removed from the refrigerator and warmed to room temperature.
18. Micro-well strips were washed as in step 13.
19. 100 μ L of Substrate Solution was added to each well and incubated at room temperature on an orbital shaker.
20. The plate was watched carefully for color changing for 2-10 min. It was stopped when the standard curve wells showed different color gradations.
21. The enzyme reaction was stopped by adding 100 μ l of Stop Solution into each well.
22. Rat's plasma absorbance was read immediately using the spectrophotometer at 450 nm.
23. The 8-OHdG content in unknown samples was determined by comparison with the 8-OHdG standard curve.

Standard Tubes	8-OHdG Standard (μ l)	Assay Diluent (μ l)	8-OHdG (ng/ml)
1	10	990	20
2	500 of additional tube that has 500 of tube #1+ Assay Diluent	500	5
3	500 of tube #2	500	2.5
4	500 of tube #3	500	1.25
5	500 of tube #4	500	0.625
6	500 of tube #5	500	0.313
7	500 of tube #6	500	0.156
8	500 of tube #7	500	0.078
9	500 of tube #8	500	0

Figure 2: 8-OHdG standard curve

- **Glutathione assay**

A GSH assay kit was purchased from Sigma Aldrich. The GSH kit uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The reaction rate is proportional to the concentration of glutathione up to 2 mM. The yellow product, 5-thio- 2-nitrobenzoic acid (TNB) is measured spectrophotometrically at 412 nm. The assay uses a standard curve of reduced glutathione to determine the amount of glutathione in the biological sample (Sigma Aldrich product manual).

I prepared the rat's red blood cells immediately after cardiac puncture as following:

1. First, I prepared the working solutions:
 - 5-sulfosalicylic acid (5-SSA) 2.5 g was dissolved in 50 ml of water.
 - PBS was prepared by adding 200 ml of water to a PBS tablet.
2. PSB was added three times the volume of red blood cells in the tube and centrifuged at 600 x g at 4°C for 10 minutes.
3. The supernatant was removed and PSB was added again and centrifuged at 600 x g at 4 °C for 10 minutes.
4. The supernatant was removed again and 200 µl of the red cells (bottom) was added to 200 µl of 5-SSA in centrifuge tubes.
5. All tubes were vortexed and kept for 10 min at 4 °C.
6. After that the tubes were centrifuged at 10,000 x g in the cold room for 10 minutes.
7. 20 µl of the top solution was diluted 10 fold with Assay Buffer.
8. Red blood cells samples were frozen at -80 °C until assayed.

Before starting the assay I did a practice test and several evaluations of red blood cells from six extra rats to determine the best dilution for the standard curve and to determine the best volume of the sample needed to start the reaction. I found that a 10 fold dilution and 6 µl of the sample after dilutions is the best to use in the assay. I also found out that the reaction and the color change very quickly. Therefore, I needed to separate the rats into five plates, each containing one or two rats from each group at a time to avoid pipetting delay.

After few days from collecting blood, GSH assay stock solutions were prepared as follows:

1. Assay Buffer was prepared by adding 4.8 ml of it to 19.2 ml distilled water.
2. Enzyme Solution was prepared by adding 3.8 μ l of Glutathione Reductase to 246.2 μ l Assay Buffer.
3. Working Mixture was prepared by the addition of 8 ml of Assay Buffer, 228 μ l and 228 μ l of DTNB Stock Solution with mixing thoroughly.
4. NADPH Solution was prepared by adding 10 μ l NADPH stock solution to 2.5 ml Assay Buffer.

GSH assay steps:

1. Spectrophotometer was set to 412 nm as kinetic assay, which uses the slope of the sample, at 10 minutes with shaking every 20 seconds.
2. Rats' red blood cells were divided into five separate plates and each group was removed from the freezer 30 minutes before the assay started.
3. To avoid pipetting errors blood cells were transferred to the wells as follows: 24 μ l of blood cells were added to 16 μ l of 5-SSA and then I took 10 μ l of the working mixture to wells in duplicates.
4. Glutathione Standard Curve Solutions were prepared in duplicate (Table 2).
5. Two blank wells were created by adding only 10 μ l of 5-SSA to each well.

6. 150 μl of the Working Mixture was added to all wells with a repeating pipette and incubated for 5 min at room temperature.
7. 50 μl of NADPH Solution was added to all wells with a repeating pipette and mixed in an orbital shaker for 1 min.
8. The Spectrophotometer was used to measure the absorbance in rat's red blood cells.

Standard Tubes	GSH Concentration (μl)	GSH Solution (μl)	5% SSA (ng/ml)	nmoles GSH in a 10 μl sample
1	50	50	None	0.5
2	25	25 from tube #1	25	0.25
3	12.5	25 from tube #2	25	0.125
4	6.25	25 from tube #3	25	0.0625
5	3.125	25 from tube #4	25	0.0312
6	1.625	25 from tube #5	25	0.015
7	0.812	25 from tube #6	25	0.007
8	0.406	25 from tube #7	25	0.003
9	0.203	25 from tube #8	25	0.001
10	0.10	25 from tube #9	25	0

Figure 3: GSH standard curve preparation

Total GSH was calculating from the equation:

$$\frac{\text{nmoles GSH}}{\text{per ml of sample}} = \frac{\Delta A_{412}/\text{min}(\text{sample}) \times \text{dil}}{\Delta A_{412}/\text{min}(1 \text{ nmole}) \times \text{vol}}$$

D A412/min (sample) = slope generated by sample (after subtracting the values generated by the blank reaction).

D A412/min (1 nmole) = slope calculated from standard curve for 1 nmole of GSH

dil = dilution factor was 10 fold

vol = volume of sample in the reaction was 6 μ l which is 0.006 ml.

I used the values of the Glutathione Standard Solutions equivalent to 1 nmole of reduced glutathione per well.

- **Rats' body weights**

Rats' body weights were measured weekly for 12 weeks. The weight gain was calculated by subtracting the starting weight from the last weight.

- **Statistical analysis**

For 8-OHdG, a mean of duplicate wells was used for each rat. Data were collected from two different immunoassay plates, each with its own standard curve, with half the rats from each group on each plate. Therefore, I did a two-sample t-test on the transformed data from the two plates to see if the plates differed significantly. I found that there was no significant difference between the two plates. 8-OHdG data did meet the criteria for parametric tests because the data were normally distributed. A one way

ANOVA test was used, followed by Pairwise Multiple Comparison Procedures (Fisher LSD Method) with α set at 0.05. This test was applied using Sigma Plot software.

For GSH a mean of triplicate wells was calculated for each animal. The data were collected from five different plates because the reaction and the color changed very quickly. Because there always are slight variations among standard curves, data could not be combined from the five plates for a parametric test. Therefore, the Kruskal-Wallis test was used with α set at 0.05. This test was applied using InStat software.

Body weight data were normally distributed so one way ANOVA was used, followed by Pairwise Multiple Comparison procedures (Fisher LSD Method) with α set at 0.05. This test was applied using Sigma Plot software.

Results

Tumors were found in only two DMBA treated rats. Both tumors were near the axilla (Figure 4). However, there was a coloration observed in the mammary glands in four of the other DMBA treated rats. A dark red to black color was observed in the mammary gland position (Figure 5). Therefore, six of the 10 DMBA treated rats had changes in the mammary glands, but none of the rats in the other groups did.

Three TQ treated rats died because of drug administration errors. TQ was mixed with oil and that caused the gavage needle to become slippery. Unfortunately, after

administering the dose three TQ treated rats were having difficulty breathing and died eventually.



Figure 4: Mammary tumor in one of the DMBA treated rats. The tumor was found near the axilla after dissecting the rat.

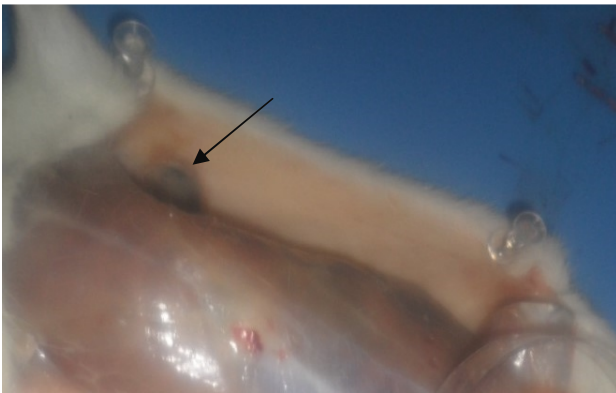


Figure 5: Mammary gland coloration in one of the DMBA treated rats. The coloration was found after dissecting the rat. The black arrow shows the coloration position.

One way ANOVA test showed that plasma concentration of 8-OHdG differed significantly among groups ($P= 0.002$). Pairwise Multiple Comparison Procedures (Fisher LSD Method) showed that 8-OHdG was significantly higher in the TAM group compared with the TQ ($P < 0.001$), the control ($P= 0.008$) and the DMBA ($P=0.041$) groups. TQ significantly decreased 8-OHdG by itself compared with DMBA ($P= 0.039$) and TAM, but did not significantly reduce the effect of TAM in the TAM+TQ group. The TAM+TQ group was significantly higher than the TQ group ($P= 0.002$) (Figure 6).

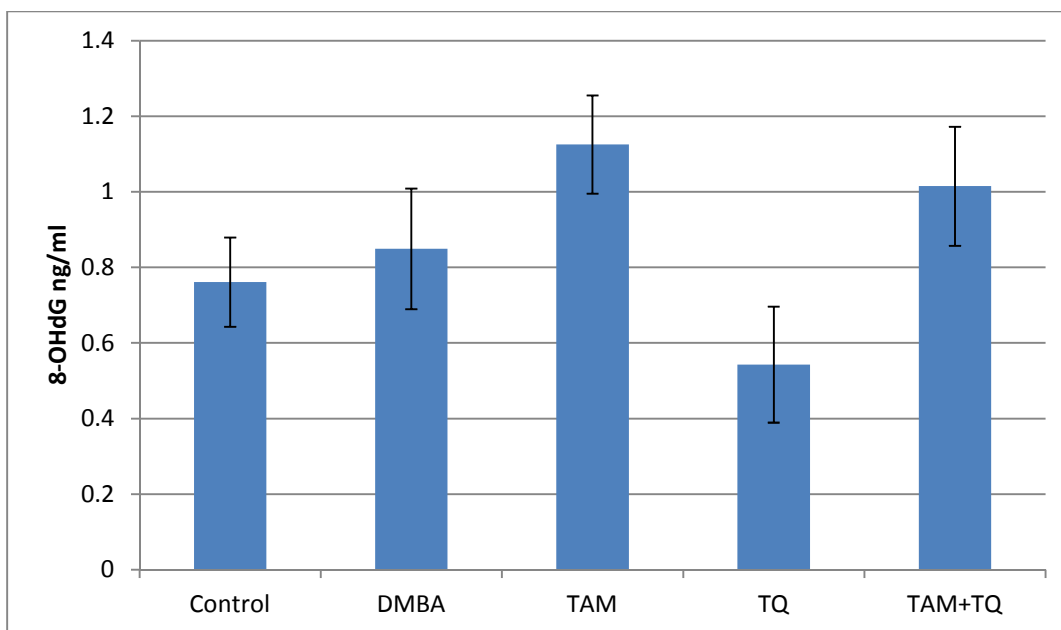


Figure 6: Values are means \pm S.D. for 8-OHdG levels in Control, DMBA, Tamoxifen (TAM), Thymoquinone (TQ), and TAM+TQ groups. 8-OHdG levels depend on plasma absorbance at 450 nm using a spectrophotometer. 8-OHdG data showed a significant difference among the groups ($P= 0.002$) using the One Way ANOVA test. 8-OHdG was significantly higher in the TAM group compared with the TQ ($P <0.001$), the control ($P= 0.008$) and the DMBA ($P=0.041$) groups. TQ significantly decreased 8-OHdG by itself compared with DMBA ($P= 0.039$) and TAM. The TAM+TQ group was significantly higher than the TQ group ($P= 0.002$) using Pairwise Multiple Comparison Procedures.

In contrast, the Kruskal-Wallis test for GSH in red blood cells showed that GSH levels did not differ among the groups ($P=0.7$). Therefore TQ did not change GSH levels by itself or combined with TAM (Figure 7).

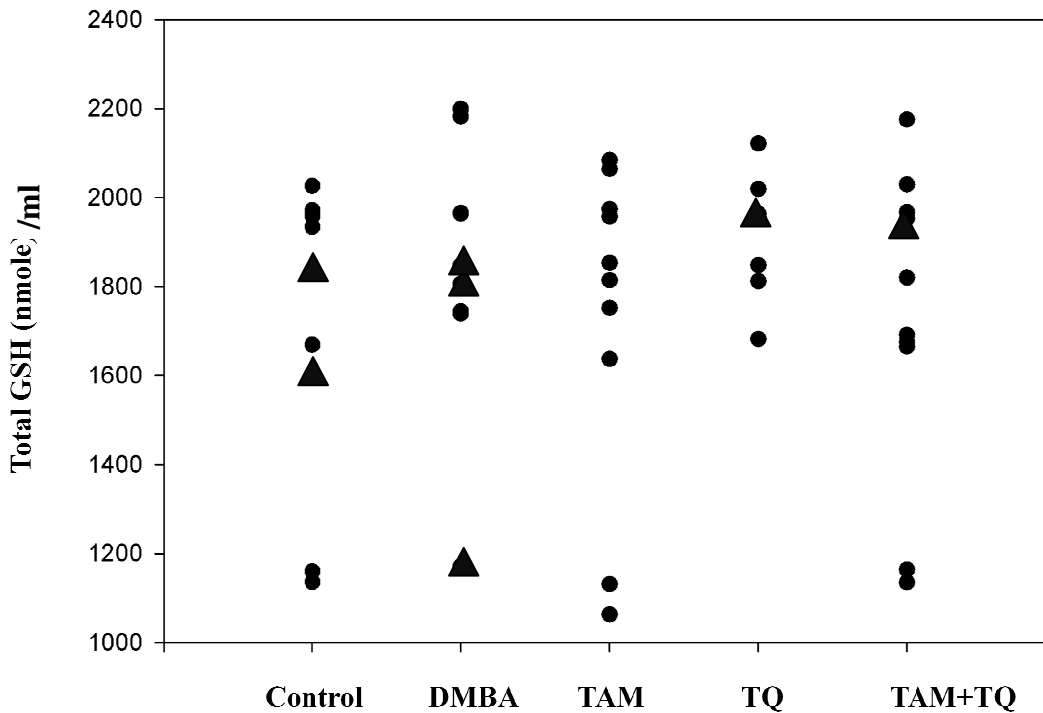


Figure 7: Total glutathione (nmole /ml) in Control, DMBA, Tamoxifen (TAM), Thymoquinone (TQ), and TAM+TQ groups. There was no significant difference among the groups ($P= 0.7$) using Kruskal-Wallis test. (\blacktriangle) shape represents two times repeated value.

On the other hand, the one-way ANOVA for gain in body weight over 12 weeks showed a significant difference among the groups ($P<0.001$). The results from Pairwise Multiple Comparison procedures showed that the TAM group had a lower weight gain compared with the TQ ($P=0.012$), the control ($P<0.001$) and the DMBA ($P< 0.001$) treated groups. Also the TAM+ TQ group showed significantly less weight gain compared with the control ($P< 0.001$), the TQ ($P= 0.002$) and the DMBA ($P< 0.001$)

treated groups. In contrast, the TQ group did not differ from the controls and the DMBA groups (Figure 8).

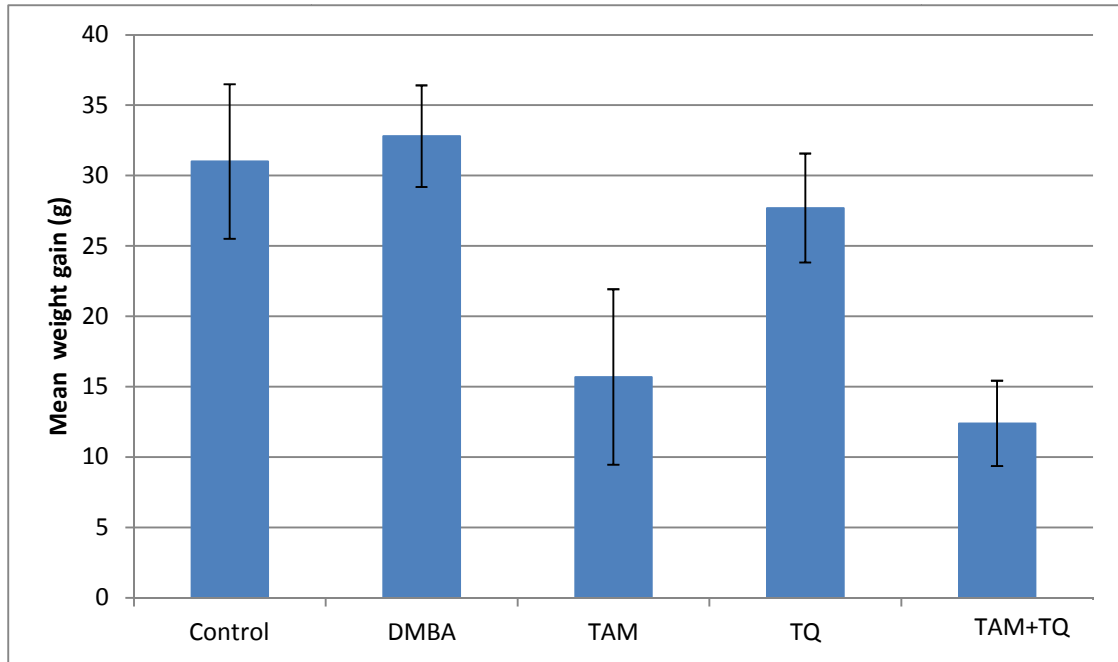


Figure 8: Values are means \pm S.D. for weight gain over 12 weeks among the groups: Control, DMBA, Tamoxifen (TAM), Thymoquinone (TQ), and TAM+TQ. ANOVA showed a significant difference among the groups ($P < 0.001$). The TAM group showed significantly less weight gain compared with the TQ ($P = 0.012$), the control ($P < 0.001$) and the DMBA ($P < 0.001$) treated groups. Also the TAM+ TQ group showed significantly less weight gain compared with the control ($P < 0.001$), the TQ ($P = 0.002$) and the DMBA ($P < 0.001$) treated groups.

Discussion

Tumors were found in only two DMBA treated rats but there was a dark coloration observed in the mammary glands of another four rats in this group. Rao & Das (1985) reported that when 20 mg of DMBA was given to rats that were maintained on normal diet for 32 weeks, approximately 70% of the animals developed mammary tumors. They also mentioned that the addition of aminoglutethimide to animals' diets reduced the number of tumors. In my study, if we counted the tumors and the coloration in the mammary glands together, 60% of the DMBA treated rats developed mammary gland changes in only 12 weeks. However, there were no tumors or colorations in the rest of the groups. Thus, there is a possibility that the TAM, TQ and TAM+TQ treated groups had no tumors because of the protective role of these treatments.

TQ has been reported to be effective in treating cancer. Woo et al. (2011) investigated the effect of TQ on breast cancer cells. They found that TQ suppressed the growth of all breast cancer cell lines in a dose- and time-dependent manner. In addition, the combination of TQ and Doxorubicin (drug used for cancer) was studied by Effenberger-Neidnicht and Schobert (2011) and showed that TQ improved the anticancer properties of doxorubicin in a cell line-specific manner. Moreover, TAM is the most widely prescribed drug for the prevention and the treatment of breast cancer (Curtis, 2001). My study provides more evidence that both TQ and TAM may be effective in preventing the development of cancer.

In my study 8-OHdG was significantly higher in rats' plasma in the TAM group compared with the TQ ($P < 0.001$), the control ($P = 0.008$) and the DMBA ($P = 0.041$) groups. The National Institute for Environmental Health Sciences listed substances that are known to cause cancer and Tamoxifen was one of them (New York Times, 2000). It was known to cause uterine cancer (Grady, 2013). In my study TAM significantly increased 8-OHdG compared with TQ, which may be a reason that TAM induces different types of cancer. TQ could be a better anticancer drug than TAM because it decreases 8-OHdG. My result suggested that TQ could be a promising natural, healthy nutrient that protects against 8-OHdG which is the most commonly assayed fingerprint of free radical attack towards DNA. 8-OHdG is also known to be involved in tumor progression (Marnett, 2002; Wiseman and Halliwell, 1996).

Notably, TAM has been reported to increase DNA damage not only in cancer cells but also in normal cells. Wozniak et al. (2007) compared the DNA-damaging potential of TAM in MCF-7 breast cancer cells and normal human peripheral blood lymphocytes. They found that TAM damaged DNA in normal and cancer cells, causing DNA strand breaks. In my study TAM increased 8-OHdG compared with the control, DMBA and TQ groups.

TQ has been reported to protect normal cells from DNA damage. A study by Rastogi et al. (2010) investigated the protective effect of *N. sativa* against radiation-induced oxidative damage. They found a significant prevention of the formation of lipid-peroxides and intracellular ROS. Moreover, the prevention of DNA damage was

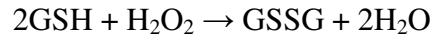
observed. In my study I am suggesting that the active component of *N. sativa* is TQ, which reduced 8-OHdG compared with TAM and DMBA groups.

In another study, TQ by itself showed a selective effect toward cancer cells. Gurung et al. (2010) investigated the cytotoxic effect of TQ in brain tumor and normal cells. TQ caused DNA damage, cell cycle arrest and apoptosis in the glioblastoma (brain cancer cells) only, but not in normal cells. Thus, TQ did not cause any damage to normal cells. Another study by Woo et al. (2011) reported for the first time that TQ was able to increase PPAR- γ activity which down-regulates the expression of the genes for Bcl-2, Bcl-xL and decreases the survival of breast cancer cells. Therefore, TQ appears to have a protective role toward the whole body by causing DNA damage in cancer cells and protecting normal cells, unlike TAM which causes DNA damage in both normal and cancer cells. In my study there was no difference in 8-OHdG levels between the TQ and the controls but TQ decreased 8-OHdG compared with TAM and DMBA groups.

Conversely, total glutathione (GSH) in red blood cells did not differ among the groups ($P=0.7$). GSH helps to protect cells from ROS, free radicals and peroxides (Pincemail et al., 2001). In fact, TAM has been observed to reduce GSH production. A study by Bruning et al. (2010) observed a significant reduction of the GSH level by 7% for MCF7 (cancer cells) treated with TAM. However, in my study total GSH in red blood cells did not differ from other groups. This is could be because Bruning et al. used TAM on cancer cells and they studied GSH at a molecular level rather than as total GSH in red blood cells. On the other hand, glutathione peroxidase (GPX) was reported to be significantly

increased compared to controls in rats with colon cancer treated with TQ (Harzallah et al., 2012).

The main role of GPX is to protect an organism from oxidative damage by catalyzing the reaction:



in which the reduced GSH forms glutathione disulfide (GSSG). GSH+ GSSG constitute the total GSH in the body. In the Harzallah et al. (2012) study, GPX level increased compared to controls in rats with colon cancer treated with TQ. In my study total GSH was not affected by TQ. The reason for that could be because GPX catalyzes other reactions and did not affect total GSH levels. Also it could be specific to colon cancer, not to mammary cancer.

TQ has been reported to enhance the performance of other cancer drugs. Effenberger-Neidnicht and Schobert (2011) investigated the influence of TQ on side effects exerted by doxorubicin (drug used for cancer) in human cancer cells. TQ improved the anticancer properties of doxorubicin in a cell line-specific manner. Effenberger-Neidnicht and Schobert found significantly more growth inhibition by doxorubicin in combination with TQ. However, in my study TQ did not decrease the 8-OHdG caused by TAM in the TAM+TQ treated rats. That could be because at the cellular level it is easier to see the effect of TQ on cancer cells compared with normal cells. In my study I evaluated the 8-OHdG and GSH in the whole body. Future studies are needed to determine the effect of TQ combined with TAM at a cellular level on 8-OHdG and GSH.

Both groups treated with TAM gained less weight than rats in the other three groups. In contrast the TQ group did not differ from the control and DMBA groups. This indicated that TAM was reducing growth in rats. However, TQ did not reduce growth by itself but did not prevent the failure of weight gain caused by TAM in TAM+TQ treated group. According to Otis Brawley (2009), chief medical officer in the American Cancer Society, about 10 % of women taking TAM report unexpected weight gain. However, the psychological factor plays an important role in human weight gain. In my study I showed that TAM causes reduced weight gain after 10 weeks of treatment in female rats. In contrast, TQ did not affect weight gain like TAM, but did not reduce the effect of TAM in TAM+TQ treated rats.

Conclusion

The purpose of my study was to determine the role of thymoquinone in improving 8-OHdG and GSH compared with tamoxifen in DMBA treated female Sprague-Dawley rats. Another purpose was to investigate whether TQ could reverse a negative effect of TAM. My study showed that that 8-OHdG was significantly higher in TAM group compared with the TQ ($P < 0.001$), the control ($P = 0.008$) and the DMBA ($P = 0.041$) groups. Therefore, TQ could be a better anticancer drug than TAM because it decreases 8-OHdG. Moreover, both TAM and TQ appeared to protect the rats from mammary changes. On the other hand, total GSH did not differ among the groups ($P = 0.7$). In addition, TQ did not reduce growth like TAM. Therefore, TQ was shown to be effective in protecting from 8-OHdG and growth retardation. Further work is needed to determine how exactly TQ works on cancer cells and normal cells compared with TAM and other anticancer drugs on oxidative DNA damage.

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