PROTECTIVE EFFECT OF THYMOQUINONE ON THE LIVER TISSUES OF 7, 12-DIMETHYLBENZ (A) ANTHRACENE INDUCED EXPERIMENTAL BREAST CANCER RATS

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ABSTRACT

Objective: Thymoquinone (TQ), the main active ingredient of the volatile oil isolated from the *Nigella sativa* seeds has been shown to possess a wide array of pharmacological effects. Recently, we have reported the anticancer potential of TQ in 7, 12-dimethylbenz (a) anthracene (DMBA) induced rats. DMBA acts as a potent site and organ-specific carcinogen by generating various reactive metabolic intermediates leading to oxidative stress. The present study was hypothesized to explore the protective effect of TQ against DMBA induced liver injury in rats.

Methods: DMBA was used to induce breast cancer in rats. Oral treatment of TQ (25 mg/kg body weight) to DMBA induced rats daily for 4 weeks was found to be effective against DMBA induced mammary gland carcinogenesis in female Wistar rats. The levels of nucleic acids, oxidative stress mark and antioxidants were determined. The activities of Phase I and Phase II enzymes, tricarboxylic acid (TCA) cycle enzymes were assayed.

Results: The increased levels of DNA and RNA were found to be decreased on treatment with TQ. The altered activities of Phase I and Phase II biotransformation enzymes were found to be regulated on treatment with thymoqionone. The hepatoprotective nature of TQ was assessed by analyzing the markers of oxidative stress, and antioxidant competence in DMBA induced rats. Treatment with TQ revealed a significant decline in the levels of lipid peroxides, and a significant improvement in the levels of enzymatic and non-enzymatic antioxidants in the liver tissue. In addition, TQ modulated the activities of TCA cycle enzymes.

Conclusion: The results of the present study clearly indicate that TQ protects the tissues from oxidative stress-mediated damage which is evident from improved antioxidant status.

Keywords: 7, 12-dimethylbenz (a) anthracene, Thymoquinone, Oxidative stress, Liver tissues, Antioxidant potential.

INTRODUCTION

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. According to recent statistics, cancer is the second most common cause of death after heart disease [1]. In India, breast cancer is the most common cancer with an estimated 1, 15, 251 new diagnoses and the second most common cause of cancer-related deaths with 53,592 breast cancer deaths in 2011 [2].

Ubiquitous chemical compounds such as polycyclic aromatic hydrocarbons (PAHs) are established cancer initiators that bioaccumulate and persist in the environment, the major sources being automobile exhaust, cigarette smoke, oil furnaces and charbroiled food [3]. These PAHs are metabolized and transformed into DNA-attacking electrophiles in the body, producing PAH-DNA adducts which are found in human breast tumors [4]. This leads to the generation of highly reactive and toxic free radicals *in vivo*. The synthetic PAH, 7, 12-dimethylbenz (a) anthracene (DMBA) initiates the production of free radicals that induce carcinogenesis in rodents which mimic human cancers morphologically and histologically [5]. Therefore, DMBA induced mammary carcinogenesis is an ideal model to study the therapeutic effect of natural and synthetic agents in experimental animals.

Several studies have focused on the toxic effects of DMBA on biochemical and antioxidant parameters in the liver [6-8]. The metabolic activation and detoxification of DMBA *in vivo* are known to occur primarily in the liver and also in a variety of other organs including the mammary gland. The metabolism of DMBA in the liver often quantitatively predominates over organ-specific metabolism [9].

Although many drugs are commercially available for the treatment of breast cancer, none is found to be ideal due to undesirable side effects. This situation warrants the need for new chemotherapeutic agents with a minimum side effect for the treatment of breast cancer.

Thymoquinone (TQ) is known to be the primary active constituent of *Nigella sativa* seeds responsible for its medicinal effects and also showing promise for treatment of cancer. TQ has been reported for a wide array of pharmacological activities such as anti-inflammatory, antioxidant, and anti-neoplastic effects both *in vitro* and *in vivo* [10-12]. More recently we have reported the anticancer effect of TQ in breast cancer rats [13]. The present study is aimed to analyze the beneficial role of TQ in modifying the hepatic mitochondrial enzyme system with respect to lipid peroxidation (LPO), antioxidant status, major citric acid cycle enzymes during DMBA-induced mammary cancer in rats.

Experimental animals

Healthy female Sprague-Dawley Wistar rats, at the age group of 45-48 days were used for present investigation. Rats were housed spaciously in individual cages and maintained under standard experimental conditions: Temperature 25±1°C, relative humidity 60±5% and 12±1 hrs (light/dark cycle) in Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai - 600 113, Tamil Nadu, India. The animals were fed with commercially available balanced pellet diet (Amrut laboratory Animal Feed, Bangalore, India) and water *ad-libitum*. The animals were acclimatized for 1 week before the initiation of experiments. The experimental design was performed in accordance with the current ethical norms approved by the Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC.No:01/02/2013).

MATERIALS AND METHODS

Induction of breast cancer

A single dose of DMBA (20 mg/kg/rat) diluted in olive oil was given orally. Animals were monitored periodically. After 13 weeks of experimentation, all animals were sacrificed.

Experimental design

The rats were divided into four groups each comprising six rats as detailed below:
• Group I: Control rats
• Group II: DMBA induced rats
• Group III: DMBA+TQ
• Group IV: TQ alone.

All animals were fasted overnight and sacrificed by sodium pentothal anesthesia followed by cervical decapitation. Blood was collected with and without anticoagulant, and the serum was centrifuged at 5000 rpm for 15 minutes to obtain a clear supernatant and stored at –70°C until its use for further biochemical analysis. Liver tissues from control and experimental groups of rats were immediately excised, washed in ice-cold PBS to remove the blood stains, blotted, weighed and homogenized in Tris-HCL buffer (0.1 M, pH 7.4) using a Teflon homogenizer to prepare 10% (w/v) tissue homogenate. This homogenate was centrifuged at 12,000 g for 30 minutes at 4°C to obtain a clear supernatant. This supernatant was pooled and used for further analysis.

Biochemical analysis

Nucleic acids from breast tissues were extracted by the method of Schneider [14], and the DNA and RNA were quantified by the method of Burton and Rawal et al., respectively [15,16].

Estimation of phase I and phase II enzymes

Phase I detoxification enzymes such as cytochrome P450 and cytochrome b_5 were assessed [17]. Phase II detoxification enzymes such as glutathione (GSH) S-transferase (GST) and UDP glucuronyl transferase (quinone reductase) were also measured [18,19]

Assay of tricarboxylic acid (TCA) cycle enzymes

The major citric acid cycle enzymes such as isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and α-ketoglutarate dehydrogenase were estimated [20-23].

Oxidative stress markers and antioxidant assays

The levels of lipid peroxides, hydroperoxides, and protein carbonyls were also determined [24-26]. The activities of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and GSH peroxidase (GPx) were assayed in the tissue homogenate of control and experimental groups of rats [27-29]. The levels of nonenzymatic antioxidants, vitamin C, vitamin E, and GSH were determined [30-32].

Statistical analysis

The results were expressed as mean±standard deviation of six rats per group and the statistical significance was evaluated by one-way analysis of variance using the SPSS (Version 16) program followed by LSD.

Effect of TQ on the levels of nucleic acids control and experimental groups of rats. Tumor induced rats showed a significant increase in nucleic acid content in the liver tissue compared to control animals (Table 1). However, on the administration of TQ to DMBA induced rats. The levels of nucleic acids in liver tissue were found to be significantly

decreased. The nucleic acid levels remained the same in control and TQ alone treated animals.

Effect of TQ on the mitochondrial TCA cycle enzymes in the liver of control and experimental groups of rats

The effect of TQ on the mitochondrial TCA cycle enzymes in the liver of control and experimental animals are showed in Table 2. A significant decrease in the levels of TCA cycle enzyme was observed in DMBA induced rats when compared to control animals. However, the levels of TCA cycle enzymes are significantly increased in TQ treated rats when compared to DMBA induced rats. No changes in drug control rats were observed when compared with control animals.

Effect of TQ on phase I and phase II enzymes in the liver of control and experimental groups of rats

The effect of TQ on the activities of Phase I and Phase II drug metabolizing enzymes in liver microsomes of control and experimental animals are presented in Table 3. In DMBA induced rats, the levels of Phase I enzymes such as cytochrome P450, cytochrome b5, and NADPH cytochrome C reductase were decreased when compared with control rats. Interestingly Phase II biotransformation enzymes such as UDP glucronyl tranferase were significantly increased. However, glutathione-S-transferase was significantly decreased in DMBA induced rats when compared with control. These altered xenobiotic enzymes were brought back to near standard level in TQ treated rats. No difference was noted in drug control rats when compared to control rats.

Effect of TQ on the levels of oxidative stress markers in liver tissue of control and experimental animals

The effect of TQ on the levels of lipid peroxides, hydroperoxides and protein carbonyls, respectively, in the hepatic tissues of control and experimental groups of rats were represented in Table 4. The levels of oxidative stress markers in DMBA induced rats were significantly escalated when compared with control rats. However, TQ treated rats demonstrated a marked decrease in these levels. No considerable statistical variation was experienced in the drug control rats.

Effect of TQ on the levels of enzymatic antioxidants in liver tissue of control and experimental animals

The activities of enzymatic antioxidants in mammary tissue are shown in Table 5. The levels of SOD, CAT and GPx were significantly lowered in tumor-induced rats compared to control animals. Oral treatment with TQ to DMBA induced rats improved the enzymatic antioxidant levels to near normalcy. However, the administration of TQ alone to animals did not cause any significant change in the antioxidant levels compared to control animals.

Activity is expressed as: 50% of inhibition of epinephrine autoxidation/min/mg of protein for SOD; µM of hydrogen peroxide decomposed/min/mg of protein for catalase; µM of glutathione oxidized/min/mg of protein for GPx.

Effect of TQ on the levels of non-enzymatic antioxidants in liver tissue of control and experimental animals

The effect of TQ treatment on the activities of nonenzymatic antioxidants in liver tissue is represented in Table 6. The levels of vitamin C, vitamin E and GSH were significantly decreased in DMBA induced rats compared to control. Oral administration of TQ to tumor bearing animals significantly increased the non-enzymatic antioxidant levels to near normalcy. However, the administration of TQ to control

Table 1: Effect of TQ on the levels of nucleic acids in liver tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
DNA (mg/g wet tissue)	6.04±0.18	9.42±0.79[a]*	7.90±0.43[a]*[b]*	5.96±0.18[aNS]
RNA (mg/g wet tissue)	3.56±0.10	5.52±0.55[a]*	4.62±0.31[a]*[b]*	3.45±0.020[aNS]

Values are given as mean±SD for groups of six rats in each. Statistical significance was compared within the group as follows: a: Group II, III, IV compared with Group I, b: Group III compared with Group II, *p<0.05, , NS: Not significant, TQ: Thymoquinone, DMBA: 7,12-dimethylbenz (a) anthracene, SD: Standard deviation

Table 2: Effect of TQ on the levels of TCA cycle enzymes in liver tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
Isocitrate DHase (n mol of α-ketoglutarate formed/mg protein/minutes)	4.06±0.20	3.78±0.46[a*]	4.82±0.22[a*b*]	4.69±0.17[aNS]
Succinate DHase (μ mol of succinate oxidized/mg protein/minutes)	7.30±0.51	5.15±0.74[a*]	7.46±0.43[a*b*]	7.87±0.40[aNS]
Malate DHase (μ mol of NADH oxidized/mg protein/minutes)	7.10±0.56	4.62±0.47[a*]	5.95±0.46[a*b*]	6.46±0.30[aNS]
α Ketoglutarate DHase (μ mol of potassium ferrocyanide liberated oxidized/mg protein/minutes)	1.73±0.29	1.09±0.09	1.66±0.10	1.48±0.08[aNS]

Values are given as mean±SD for groups of six rats in each. Statistical significance was compared within the group as follows: a: Group II, III, IV compared with Group I, b: Group III compared with Group II, *p<0.05 NS: Not significant, TQ: Thymoquinone, DMBA: 7,12-dimethylbenz (a) anthracene, TCA: Tricarboxylic acid, SD: Standard deviation

Table 3: Activities of Phase I and Phase II biotransformation enzymes in liver tissues of control and experimental groups of rats

Groups	Control	DMBA	DMBA+TQ	TQ
Phase I enzyme (cytochrome P450 n moles/mg microsomal protein/minutes)	0.80±0.04	0.40±0.039[a*]	0.60±0.035[a*b*]	0.85±0.041[aNS]
Cytochrome b5 (n moles/mg microsomal protein/minutes)	0.75±0.029	0.31±0.039[a*]	0.40±0.035[a*b*]	0.70±0.019[aNS]
NADPH cytochrome 'C'Reductase (n moles/mg microsomal protein/minutes)	17.40±0.80	12.01±2.13[a*]	14.10±1.06[a*b*]	16.09±0.24[aNS]
Phase II enzyme GST (n moles/mg microsomal protein/minutes)	2.87±0.096	3.36±0.80[a*]	4.08±0.22[a*b*]	2.76±0.087[aNS]
UDP glucronyl tranferase (n moles/mg microsomal protein)	35.72±0.096	48.09±4.60[a*]	42.70±2.89[a*b*]	34.18±1.96[aNS]

Values are given as mean±SD for groups of six rats in each. Statistical significance was compared within the group as follows: a: Group II, III, IV compared with Group I, b: Group III compared with Group II, *p<0.05, NS: Not significant, TQ: Thymoquinone, DMBA: 7,12-dimethylbenz (a) anthracene, SD: Standard deviation, GST: Glutathione S-transferase

Table 4: Effect of TQ on the levels of oxidative stress markers in liver tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
TBARS	1.70±0.20	5.00±0.35[a*]	2.34±0.22[a*b]	1.51±0.16[aNS]
Hydroperoxides	60.14±2.21	99.90±8.80[a*]	77.10±3.51[a*b]	60.91±1.90[aNS]
Protein carbonyls	5.51±0.17	19.91±2.00[a*]	10.92±0.80[a*b]	5.11±0.22[aNS]

Values are given as mean±SD for groups of six rats in each. Statistical significance was compared within the group as follows: a: Group II, III, IV compared with Group I, b: Group III compared with Group II, *p<0.05, NS: Not significant, TQ: Thymoquinone, DMBA: 7,12-dimethylbenz (a) anthracene, SD: Standard deviation, TBARS: Thiobarbituric acid reactive substances

Table 5: Effect of TQ on the levels of enzymatic antioxidants in liver tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
SOD	8.19±0.60	5.60±0.62[a*]	6.19±0.20[a*b*]	7.53±0.23[aNS]
CAT	57.05±3.89	33.10±3.46[a*]	45.26±3.18[a*b*]	53.81±2.60[aNS]
GPX	6.12±0.80	3.70±0.53[a*]	4.16±0.11[a*b*]	5.22±0.18[aNS]

Values are given as mean±SD for groups of six rats in each. Statistical significance was compared within the group as follows: a: Group II, III, IV compared with Group I, b: Group III compared with Group II, *p<0.05, NS: Not significant, GPX: Glutathione peroxidase, SOD: Superoxide dismutase, CAT: Catalase, TQ: Thymoquinone, DMBA: 7,12-dimethylbenz (a) anthracene

Table 6: Effect of TQ on the levels of non-enzymatic antioxidants in liver tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
Vitamin C (μg/mg protein)	3.90±0.19	2.73±0.44[a*]	3.04±0.02[a*b*]	3.36±0.10[aNS]
Vitamin E (μg/mg protein)	3.94±0.19	3.07±0.49[a*]	3.17±0.26[a*b*]	3.50±0.40[aNS]
GSH (mg/100 g tissue)	8.75±0.50	5.71±0.74[a*]	6.26±0.24[a*b*]	8.10±0.43[aNS]

Values are given as mean±SD for groups of six rats in each. Statistical significance was compared within the group as follows: a: Group II, III, IV compared with Group I, b: Group III compared with Group II, *p<0.05, NS: Not significant, TQ: Thymoquinone, DMBA: 7,12-dimethylbenz (a) anthracene, SD: Standard deviation, GSH: Glutathione

animals did not cause any significant change in the antioxidant levels compared to control animals.

DISCUSSION

Mitochondria, the power house of the cell, are the chief target of reactive oxygen species (ROS) which plays a significant role in cellular metabolism and hence it is the source of energy during oxidative phosphorylation. Detonated production of ROS and free radicals in mitochondria results in mitochondrial DNA mutations which indirectly impair glucose sensing by reducing intracellular concentrations of adenosine triphosphate (ATP) [33]. Most cancers probably start with an interruption of the Krebs cycle that arrests aerobic metabolism and force the cells to revert back to anaerobic metabolism. DMBA induced breast tumor bearing rats showed a significant reduction in the activities of the Krebs cycle enzymes which evidences the defect in the aerobic oxidation of pyruvate that might cause the low production of ATP molecules [34]. In the present investigation, the decreased activities of TCA cycle enzymes that were observed in breast cancer bearing animals may be due to the severe damage in mitochondria due to oxidative stress. However, oral administration of TQ increased the activities of the mitochondrial enzymes activity which is due to the protective role of TQ against the DMBA induced liver damage.

The metabolism of xenobiotics is mediated by the biotransformation enzymes such as Phase I and Phase II enzymes which convert biologically

inactive compound into active or toxic metabolites [35]. Phase I enzyme catalyzes functional group of xenobiotic into the hydrophilic substrate. Phase II enzymes make the molecule less reactive by conjugation of the functional group with glutathione, sulfate or glucuronic acids. These reactions generally make the substrate into water soluble, and the conjugated endogenous compound further facilitates the excretion of the product. In the present study, the altered activities of the xenobiotic enzymes were normalized indicating the beneficial effects of TQ.

Oxidative stress markers are produced during the free radical attack on polyunsaturated fatty acids. DMBA induces the production of various free radicals such as superoxide anion, hydroxyl radical and NO that cause damage to macromolecules and trigger cell injury [36]. The markers of oxidative stress such as LPO, hydroperoxides, and protein carbonyls are produced during LPO. In the present DMBA, induction increased the levels of the free radicals in liver tissues and TQ treatment significantly scavenges them. This is due to the antioxidant and free-radical scavenging potential of TQ.

The enzymatic and non-enzymatic antioxidants play a major role in scavenging the reactive free radicals in the DMBA induced liver. The enzymatic antioxidants SOD, CAT, and GPx function as the first-line of defense against oxidative stress by virtue of their ability to catalyze the disproportionation reactions of their substrate free radicals that are spontaneously generated via *in vivo* oxidative phosphorylation, cytochrome P450 metabolism, and inflammatory processes [37]. GSH is an important non-protein cellular thiol that in conjunction with GPx plays a regulatory role in cell proliferation. GSH directly detoxifies ROS and neutralizes reactive intermediates generated from exposure to xenobiotics including chemical carcinogens. In the present study, DMBA induction notably decreases the levels of intracellular antioxidants and elevates the formation of pro-oxidants such as reactive free radicals that eventually results in liver dysfunction and deterioration. Likewise, the levels of non-enzymatic antioxidants such as vitamin C and E were improved. However, the diminished antioxidant status was normalized on TQ administration indicating the antioxidant potential of TQ.

CONCLUSION

TQ modulates the activities of mitochondrial TCA cycle enzymes and xenobiotic enzymes in DMBA induced breast cancer rats. In addition, the results of the present study indicate that TQ possesses antioxidant potential and protects the hepatic tissues from oxidative stress-mediated damage that is caused due to the toxic effects of DMBA.

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