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Carcinogen Metabolizing Enzymes: Modulation of Their Activities in Liver, Lung and Stomach by Thymoquinone

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Authors' contributions

This whole work carried out by all authors. Finally all authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Thymoquinone (TQ), the bioactive constituent of black seed (*Nigella sativa)*, has been shown to inhibit the growth of various human cancerous cells both in vitro and in vivo.
Aim: To characterize the effects of thymoquinone on the activity of phase I and phase II carcinogen metabolizing enzymes in rats.
Materials and Methods: Phase I enzymes, namely the cytochrome P450 enzymes CYP1A1 and CYP2E1, and phase II enzymes, including UDP-glucuronyltransferase (UGT) and glutathione *S*-transferase (GST), were studied in the liver, lung and stomach of female Swiss albino rats. The animals were divided into two groups (10 rats/group), a control group treated with corn oil and a TQ-treated group receiving oral (gavage) thymoquinone at a dose of 10 mg/kg/day for 15 consecutive days. Animals were then sacrificed on day 16. Tissue homogenates of liver, lung and stomach were prepared to evaluate the activities of both phase I and phase II selected enzymes.
Results: Thymoquinone treatment induced significant modulation of the selected phase I and phase II enzymes in a tissue-specific manner. Our results revealed statistically significant reductions in the activities of CYP1A1 enzyme (46%, 60% and 57% in liver, lung and stomach respectively) versus the control group. Similarly, CYP2E1 activities

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were decreased in both liver and lung, by 51% and 16%, respectively, compared to the control group. UGT enzyme showed a decrease of 51% in liver, but a significant rise in both lung and stomach, by 40% and 192%, respectively. GST activity, on the other hand, was moderately enhanced, by 24%, 50% and 30% in liver, lung and stomach, respectively.

Conclusion*:* Thymoquinone, in addition to scavenging active metabolites of chemical carcinogens, may also change their metabolisms by modulating the activity levels of enzymes involved in carcinogen activation and/or xenobiotics pathways.

Keywords: Thymoquinone; drug metabolism; black seed; Nigella sativa; herbal drugs; CYP enzymes; anticancer.

1. INTRODUCTION

Since cancer is not controlled through current prevention, early detection and treatment methods, great effort is still being exerted towards the development of effective new strategies to control the disease. One such approach currently under intense interest and investigation is the chemoprevention, a clinical strategy to block or reverse carcinogenesis before the detection of invasive cancer [1]. Recently, there has been growing interest in naturally occurring phytochemical compounds with anticancer potential as they are relatively non-toxic, inexpensive and available in ingestible forms. Certain plant-derived compounds may afford protection against the formation and action of mutagenic and carcinogen metabolites [2]. Various studies have indicated that the chemopreventive actions of certain phytochemicals can be associated with one of the following mechanisms: (a) inhibition of metabolic activation, (b) prevention of interaction between DNA and reactive metabolites, (c) enhancement of the detoxification of reactive metabolites, or (d) suppression of the mechanisms underlying tumour progress [3].

Several beneficial pharmacological effects have been attributed to various crude and purified components of black seed (*Nigella sativa*), including antihistaminergic, antihypertensive, hypoglycemic, antimicrobial, anthelmintic and antinflammatory activities [4]. Black seed preparations have also exhibited significant antineoplastic activity both in vivo and in vitro [5], and they might exert a potential chemopreventive activity against cancer as well as reducing the toxicity of standard antineoplastic drugs [6]. Thymoquinone (TQ) is the main active constituent found in the crude extracts of the seeds of *Nigella sativa*, and many studies have shown that TQ possesses antineoplastic properties [7]. TQ induces DNA damage, cell cycle arrest and apoptosis in glioblastoma cells [8]. It was also observed that TQ facilitated telomere attrition by inhibiting the activity of telomerase, and demonstrated a potent cytotoxic activity against a variety of tumour cell lines, though it was minimally toxic to normal cells [9]. Recently, it was demonstrated by Elbarbry et al. [10] that TQ has differential effects on some hepatic metabolizing enzymes in rabbits.

Before they can induce a biological response, most chemical carcinogens require metabolic activation, which consists of two successive processes known as phase I and phase II. The production of reactive metabolites is largely dependent on the primary metabolism by cytochrome P450 enzymes (phase I reaction), and the conjugation metabolism (phase II reaction), which, in contrast, is principally but not invariably detoxifying [11]. The cytochrome P450 (CYP) superfamily is a large and diverse group of enzymes that are present in most tissues of the body, that catalyze the oxidation of organic substances and that play important roles in hormone synthesis and breakdown as well as in breaking down xenobiotic

substances such as drugs and other toxic chemicals. The Human Genome Project has identified at least 57 human genes encoding for various enzymes belonging to the cytochrome P450 superfamily [12], such as CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4/5. In subsequent phase II reactions, these activated xenobiotic metabolites are conjugated with a large group of broad-specificity transferases such as UDP-glucuronyl transferase (UGT), glutathione *S*-transferase (GST) and sulfotransferases [13]. The balance between the activities of potential chemoprotective agents that can inhibit P450s and induce phase II enzymes may be critical for their capacity to function as anticarcinogen agents. The changes in phase I and phase II drug metabolizing enzymes are very important parameters by which to assess the potential of any given compound against neoplastic development [14], as they play a major role in toxic oxidative damage, mutagens, carcinogens and apoptosis regulation.

2. MATERIALS AND METHODS

2.1 Animals

Inbred mature female albino rats (Sprague Dawley strain), average weight 130-140g, were obtained from the animal facility of The Faculty of Medicine, Mutah University. They were randomly assigned to the control or treatment group, housed in cages (10/cage) and maintained under standard conditions, namely, a 12:12 hr light:dark cycle , a temperature of 24±1^{o}C, and 50±10% relative humidity. All animals were fed a standard laboratory control diet and provided with tap water *ad libitum*. The experiments were conducted according to the ethical forms approved by the Faculty Ethics Committee. The test group of animals received an oral (gavage) dose of TQ (10 mg/kg/day) for 15 consecutive days using a volume of 5 mg/kilogram body weight based on previous chemoprevention and toxicity studies [15]. The control group of animals received corn oil. Ten rats/group were sacrificed according to the current animal care regulations on day sixteen (24 hours after the final dose).

2.2 Chemicals

All chemicals, unless specified otherwise, were obtained from Sigma-Aldrich Chemical Co. (United Tetra Group, Amman, Jordan).

2.3 Preparation of Cytosolic and Microsomal Fractions

After animals were sacrificed, liver, lung and stomach were dissected. Tissue homogenates (20%) were prepared in ice-cold 0.25 M sucrose in 0.1 M Tris-HCl buffer, pH 7.4. Cytosolic and microsomal fractions were obtained through differential centrifugation (16000 g for 45 minutes and 105000 g for 45 minutes) [16]. The microsomal pellets were suspended in 0.25 M sucrose in 0.1 M Tris-HCl buffer, pH 7.4, and the protein contents of the fractions were then assayed using the method of Lowery et al. [17] with bovine serum albumin as a standard.

2.4 Cytochrome P450 1A1 (CYP1A1) Activity

CYP1A1-mediated ethoxyresorufin-O-deethylase (EROD) activities were determined by monitoring the formation of resorufin from 7-ethoxyresorufin [18]. Briefly, the assay mixture contained 0.1 phosphate buffered saline, pH 7.4, 6.25 mM $MgSo_4$, 60 µM EDTA, 5 µM

ethoxyresorufin, 100 µg microsomal proteins and 100 µM NADPH in a total volume of 1 ml. The reaction was stopped after five minutes by adding 2 ml chilled ethanol. The precipitated protein was spun down by centrifugation; the fluorescence of the supernatant was assayed fluorometrically at excitation 550 nm and emission 585 nm.

2.5 Cytochrome P450 2E1 (CYP2E1) Activity

4-nitrophenyl hydroxylase was assayed according to the method described by Reinke and Moyer [19] with slight modifications. A reaction mixture containing 0.2 mg/ml microsomes, NADPH generating system and 0.5 mM *p*-nitrophenol in 50 mM potassium phosphate, pH 7.4, was incubated at 37ºC for 30 minutes (a blank incubation was prepared using the same reaction mixture and adding microsomes immediately prior to stopping the reaction). The reaction was stopped by the addition of 20% trichloroacetic acid followed by centrifugation at 10000g for one minute. A 0.5 ml aliquot of the supernatant was added to 0.25 ml 2N NaOH and the absorbance was measured at 546 nm (with NaOH in the reference cuvette). The quantity of the product was determined by subtracting the absorbance of the blank from the absorbance of the incubation containing microsomes and calculated utilizing an extinction coefficient of 10.28 mM^{1} cm^{1}.

2.6 UDP-glucuronosyl Transferase (UGT) Activity

Microsomal UGT activity was determined fluorometrically using 4-methylumbelliferone as a substrate [20]. The assay was performed at 37^{o}C with 0.1 M Tris-HCl, pH 7.4 and 5 mM $MgCl_2$. 4-methylumbelliferone (0.5 mM final concentration) was dissolved in dimethylsulfoxide. The assay was started by the addition of 3 mM UDP-glucuronic acid and stopped by the addition of 0.5 ml of 0.5 M perchloric acid. Excessive substrate was extracted with 2 ml chloroform. After centrifugation, a 0.5 ml aliquot of the water phase containing the glucuronide was mixed with 0.5 ml of 1.6 M glycine/ NaOH, pH 10.3. The fluorescence was measured at 365 nm with excitation at 315 nm.

2.7 Glutathione S-transferase (GST) Activity

Cytosolic GST was measured spectrophotometrically by determining the rate of conjugation of 1-chloro-2, 4-dinitrobenzene as a substrate with glutathione [21]. The assay was conducted in an incubation solution containing 1 mM of 1-chloro-2,4-dinitrobenzene, 1 mM of glutathione, 100 mM potassium phosphate buffer, pH 6.5 and 0.04 mg cytosolic protein. The reaction was monitored at room temperature by measuring changes in absorbance at 340 nm over 3 min. Activity was determined from the initial linear increase in absorbance of 1-chloro-2, 4-dinitrobenzene conjugation using extinction coefficient 9.6 mM^{1} cm^{1}.

2.8 Statistical Analysis

The data are expressed as the mean±SE. Mann–Whitney–Wilcoxon test (Non-parametric test) was used as the data wasn't normally distributed based on the test result of Kolmogorov–Smirnov for the tests of normality (p-value < 0.05). Therefore, Mann–Whitney–Wilcoxon test was used to compare the two independent means of the study and control group [22]. *P* values ≤ 0.05 were considered significant.

3. RESULTS

Fig. 1 demonstrates the activity of phase I enzymes (CYP1A1 and CYP2E1) in TQ-treated rats as a percentage of the control values in corn oil-treated rats. CYP1A1 activities in liver, lung and stomach of TQ-treated rats were significantly lower than control values, by 46%, 60% and 57%, respectively. Similarly, TQ treatment significantly decreased CYP2E1 activities in liver and lung by 51% and 16%, respectively, compared to control values; in stomachs of TQ-treated rats, in contrast, the slight (10%) enhancement in CYP2E1 activity lacked statistical significance.

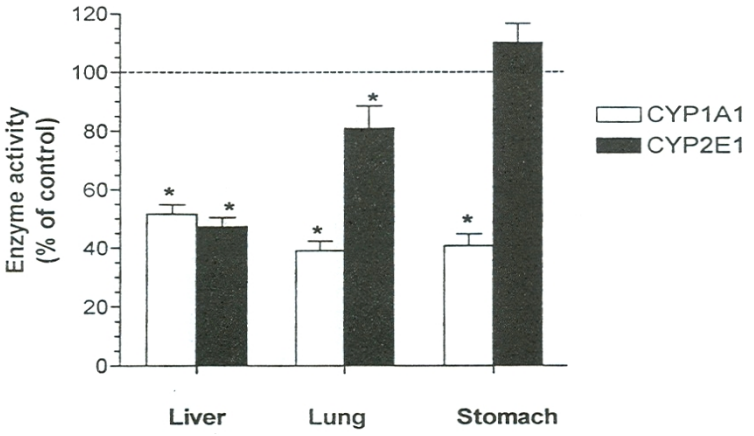

Fig. 1. Activity of phase I enzymes (CYP1A1 and CYP2E1) in liver, lung and stomach of TQ-treated rats compared to corn oil-treated rats. Values are mean±SE of 10 rats. *Indicates a significant difference from the corresponding control group at $P < 0.05$ using Mann–Whitney–Wilcoxon test.

The data presented in Fig. 2 indicate that hepatic microsomal UGT activity was significantly decreased by 51% by TQ treatment compared to the control group. TQ treatment enhanced UGT activity in the lungs (40%) and stomach (192%) compared to control values.

An assay of the specific activities of cytosolic GST in tissues of control and TQ-treated rats is presented in Table 1. GST activity was moderately enhanced, by 24%, 50% and 30% in liver, lung and stomach, respectively, in TQ-treated rats compared to control values.

Table 1. GST activities of cytosolic fractions in different tissues of TQ-treated and corn oil-treated rats

Tissue	Corn oil-treated group	TQ-treated group
Liver	1.9±0.09 (100)	2.30±0.26* (121)
Lung	0.34±0.018 (100)	0.52±0.05* (153)
Stomach	0.51±0.04* (100)	0.67±0.04* (131)

*Values are expressed as mean±SE of eight rats. Values in parentheses represent relative changes in parameters assessed. *: P<0.05 is significant when compared with the values of the control group.*

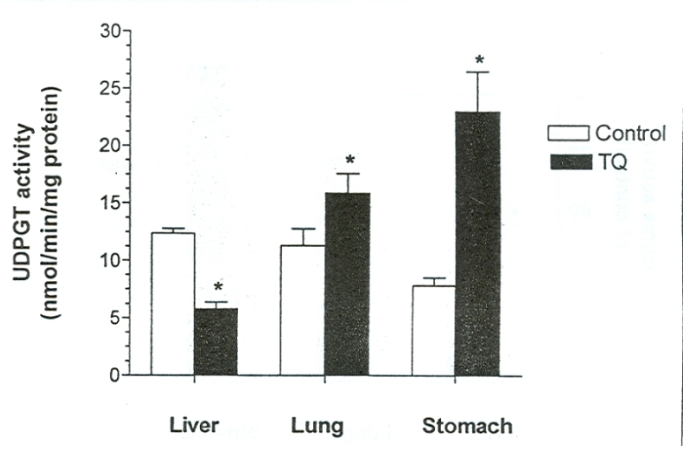

Fig. 2. Effect of TQ on UDP-glucuronosyltransferase enzyme activity in rat liver, lung and stomach. Values are mean±SE of eight rats. *Indicates a significant difference from the corresponding control group at $P < 0.05$ using Mann–Whitney–Wilcoxon test.

4. DISCUSSION

Drug metabolizing enzymes have been recognized as important markers in characterizing the metabolic pattern of preneoplastic cells and in the development of chemopreventive measures combating carcinogenesis and early-stage cancer. The aim of the present study was to evaluate the effects of naturally occurring TQ on several metabolizing enzymes in the rat liver, lung and stomach. The obtained results reveal that oral intake of TQ for 15 days could modulate some phase I and phase II enzyme activities in both hepatic and extrahepatic tissues of interest in rats. A number of naturally occurring compounds have been shown to modulate the CYP450 system by, for example, inducing specific CYP450 isozymes and activating or inhibiting these enzymes [3,10]. Many carcinogens are metabolized by CYP enzymes into either biologically inactive metabolites or chemically reactive electrophilic metabolites that covalently bind to DNA, resulting in carcinogenesis [23]. These reactive metabolites then undergo additional metabolism by phase I and II enzymes into inactive products.

TQ produced significant decreases in the activities of both CYP1A1 and CYP2E1 in all examined tissues except for CYP2E1 in the stomach. Hepatic CYP1A1 inhibition was higher than that in extrahepatic tissues. The observed variation in TQ's inhibitory effect on CYP2E1 in different tissues suggests that this effect is tissue–specific. This finding is in agreement with the results of Elbarbry et al. [10], who studied CYP1A2 and CYP3A4, although not CYP2E1; the former two were significantly diminished by TQ treatment, while the phase II enzymes glutathione-S-transferase and glutathione peroxidase were significantly induced by a high TQ dose. Glutathione reductase, on the other hand, was significantly induced by both TQ treatment and the high TQ dose. TQ has differential effects on CYP and phase II enzymes. Induction of the activity of glutathione reductase (a phase II reaction) may explain the salutatory effect of black seed in inhibiting the generation of bioactive metabolites known to promote carcinogenesis and oxidative cell damage [10].

This observation suggests a possible mechanism by which TQ reduces the activation of low molecular weight carcinogens or nitrosamines which are activated by these CYP isoforms

[24]. Given that the observed modulation is not restricted to one CYP isoform, its mechanism is probably general rather than gene-specific.

The CYP1 family consists of 1A1, 1A2 and 1B1, all of which are capable of activating procarcinogens. CYP1A1 is involved in the biotransformation of PAHs, a class of ubiquitous environmental chemicals, into carcinogen metabolites [25]. This process is thought to contribute to pulmonary cancer [26] and colorectal cancer [27]. Studies conducted in human microsomes have shown that CYP1A1 is approximately three times more active than CYP1A2 in the metabolism of 7-ethoxyresorufin [28].

CYP2E1 is the principal enzyme involved in the bioactivation of a variety of low molecular weight compounds suspected to be carcinogens, such as benzene, nitrosamines and carbon tetrachloride [29], in rodents and humans. Its activity is mainly regulated by post-transcriptional protein stabilization, but the contribution of the transcriptional step is also significant [30]. The inhibition of CYP2E1 is expected to block both the toxicity and the carcinogenicity of those compounds. Similarly, previous studies have indicated the ability of some naturally potent anticarcinogens, such as garlic [31], silymarin [32] and tannic acid [33], to inhibit CYP2E1 activity. Thus the inhibition of CYP2E1 observed in this study might play an important role in the anticarcinogen activity of TQ, perhaps through its direct inhibitory effect on P450 enzymatic activity without any effect on protein expression. TQ inhibition of CYP2E1 and CYP1A1 could result in a reduction of carcinogen activation and DNA binding.

One of the interesting outcomes in the current study was the manner in which TQ affected the two phase II enzymes (UGT and GST). These enzymes were significantly increased after TQ treatment in all tissues, except in the case of UGT in the liver, which was subjected to a dramatic inhibition of activity, confirming the hypothesis that TQ exerts tissue-specific modulation on phase I enzymes. UGTs play an important role in phase II biotransformation: they catalyze the glucuronidation of xenobiotics and endobiotics, including therapeutic drugs, carcinogens, environmental pollutants, bile acids and bilirubin, leading to their biotransformation, detoxification and elimination [34]. The TQ-mediated increases in UGT activity in the rat lung and stomach might reflect the important role of TQ in accelerating the glucuronidation reactions in these tissues. It is worth noting that the TQ inhibition of UGT conjugation activity in the rat liver might lead to the prevention of the enterohepatic circulation of drugs that are mainly metabolized by glucuronide conjugation. The coordinate inhibition of hepatic CYP1A1, CYP2E1 and UGT by TQ could be due to the involvement of a common xenobiotic and/or antioxidant response element, given the findings of Jrah-Harzallah et al. [35] concerning the efficacious role of TQ in protecting and curing the initiation phase of 1, 2-dimethyl-hydrazine-induced colon cancer while also exerting a protective role at the promotion phase of cancer by preventing oxidative stress through increased production of all ROS and lipid peroxidation during cancer initiation and promotion phases. Also, some protection against CCl4-induced hepatotoxicity in rats is most probably exerted by TQ's improving the antioxidant status of the cell through upregulation or induction of various detoxifying enzymes and GSH [36], because TQ and its metabolite dihydrothymoquinone (DHTQ) act as general free radical scavengers [37]. Mahmoud et al. [38] showed that oral administration of TQ is effective in increasing the activities of some detoxifying enzymes, including quinone reductase and glutathione transferase, and identified TQ as a promising prophylactic agent against chemical carcinogenesis and toxicity. A study on tilapia and zebrafish has shown that *N. sativa* oil and its extract TQ are effective at inhibiting chromium VI-induced mutagenicity, which may be mediated through antioxidant activity and inhibition of Chromium VI enzymatic activation, by studying the effects of *N.*

sativa oil and TQ on hepatic and brain mRNA levels of cytochromes, including CYP1A2, CYP3A and CYP2E1 [39]. Cytosolic GST is an important enzyme in hepatic and extrahepatic biotransformations [14]. It catalyzes the binding of a large variety of electrophiles to the sulfhydril moiety of GSH. Because of the electrophilic nature of reactive carcinogens, GST has received attention as one possible mechanism involved in carcinogen detoxification. The induction of GST has been postulated as the chemoprotective mechanism of a number of naturally occurring dietary constituents [40]. A clear finding of the present study was that TQ elevated GST activity in the liver, lung and stomach. This induction might increase the ability of the organism to inactivate carcinogen-derived carcinogen metabolites, thereby reducing carcinogen-DNA adduct formation and the subsequent expression of tumours. In addition, a number of previous studies have suggested that the induction of phase II enzymes is a relevant mechanism for cancer chemoprevention. Various compounds such as tannic acid [33], henna leaf Dasgupta et al. coffee components and cafestol [41] have been reported to exert broad-based anticarcinogen activity against a variety of carcinogens through the modulation of phase II enzymes as UGT and GST.

5. CONCLUSION

The results of the present study indicate that, in addition to scavenging active metabolites, TQ could modulate the activation of carcinogens and the activity of detoxifying pathways, particularly those in which CYP1A1, CYP2E1, UGT and GST are involved. To a great extent, this effect depends on the species, tissue, dose and route of administration. It is important to note that the dose chosen in the current study has been shown to modulate chemical carcinogens in rats, but that this dose is much higher than the concentration of TQ in the *N. sativa* (black seed) oil available on the market. Therefore it is not very likely that *N. sativa* oil taken as a dietary supplement would actually modulate enzymes as described here. On the other hand, until information on the safety and efficacy of TQ used in combination with other prescribed drugs is available, caution must be taken with drugs that are glucuronized for a significant part of their elimination in patients. These findings should generate demand for a detailed examination of the biochemical and molecular effects of TQ and other potential chemoprotective agents in humans.

CONSENT

Not applicable.

ETHICAL APPROVAL

The experiments were conducted according to the ethical forms approved by the Faculty Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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