Evaluation of the radioprotective effects of thymoquinone on dynamic thiol-disulphide homeostasis during total-body irradiation in rats

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(Received 28 May 2018; revised 15 August 2018; editorial decision 5 September 2018)

ABSTRACT

Ionizing radiation–induced free radicals cause functional and structural harmful effects. Thiol, an important anti-oxidant, plays a major role in the eradication of reactive oxygen molecules. Thiol/disulphide homeostasis is a marker of oxidative stress. The objective of this study was to assess the potential radioprotective effects of thymo-quinone (TQ) on the dynamic thiol/disulphide homeostasis of rats receiving total-body irradiation (IR). Twenty-two rats were divided into three groups to test the radioprotective effectiveness of TQ. The sham control group did not receive TQ or IR. The IR group received only total-body IR. The TQ + IR group received IR plus TQ. Following IR, blood samples were taken. The thiol/disulphide homeostasis parameters were analysed by a newly established method. In the IR group, native thiol and the native thiol/total thiol ratio were significantly decreased ($P = 0.003$ and $P = 0.003$, respectively), whereas the disulphide/native thiol and disulphide/total thiol ratios were significantly increased when compared with those of the sham control group ($P = 0.003$ and $P = 0.003$, respectively). In the TQ + IR group, the mean disulphide, native thiol and total thiol levels and the disulphide/native thiol, disulphide/total thiol and native thiol/total thiol ratios were not found to be significantly different when compared with those of the sham control group ($P > 0.05$ for all). Thiol/disulphide homeostasis was found to be disturbed after IR exposure. The results showed that TQ had antioxidant effects and reduced the IR-induced oxidative stress, which was demonstrated through the dynamic thiol/disulphide homeostasis. Thus, the use of TQ before radiation treatment helped protect the rats from oxidant side effects.

Keywords: dynamic thiol/disulphide homeostasis; irradiation; thymoquinone; oxidative stress

INTRODUCTION

Radiotherapy (RT) is the commonly used therapeutic modality in definitive and palliative cancer treatment [1].

The effects of RT are mediated by the production of free radicals, which induce lipid peroxidation that leads to structural and functional damage to cellular membranes. Free oxygen radicals in biological systems and other free radicals constitute one of the most significant causes of oxidative stress [2].

Thiol is an organic compound containing the sulphydryl group, and it has a critical role in preventing the formation of any oxidative stress condition in cells. Thiol groups are important members of antioxidant cascades, because the oxidation reaction of thiols with oxidizing molecules causes the formation of reversible disulphide bonds. This transformation is the earliest observable symptom of radical-mediated protein oxidation. The disulphide bond structures that form when oxidative stress conditions end can be reduced to the thiol groups again; thus, the dynamic thiol/disulphide balance, which is of vital importance for the organism, is continued. Previously, this double-sided balance could be measured on one side only, as total thiol, but today all components can be measured

separately using a newly developed method, and they can be evaluated both individually and as a whole [3].

Any tumour can be controlled by RT if a sufficient dose is delivered to all tumour cells [4]. The dose of ionizing radiation (IR) that can be given to the tumour is determined by the sensitivity of the surrounding normal tissues. Strategies to improve RT therefore aim to increase the effect on the tumour or to decrease the effect on normal tissues. This must be achieved without sensitizing the normal tissues (in the first approach) or protecting the tumour (in the second approach) [5]. Mechanisms for normal tissue injury and its related biomarkers are now being investigated, facilitating the discovery and development of a new generation of radiation protectors and mitigators [4].

Herbal medicines have attracted attention in recent years, and some are gradually being introduced as alternatives to, or are being used in combination with, chemical drugs [6].

Nigella sativa, commonly known as black cumin seed, has a long history in folk medicine, with diverse therapeutic benefits. The biological activity of this herb is primarily attributed to its main component, known as thymoquinone (TQ). TQ has antioxidant, anti-inflammatory, and anticancer effects [7].

TQ is a free radical and superoxide radical scavenger. It preserves the activity of various antioxidant enzymes [8]. TQ is a promising natural radioprotective agent against the immunosuppressive and oxidative effects of ionizing radiation [9].

The relationship between thiol/disulphide homeostasis and TQ during IR has not yet been investigated. Our aims for this study were to examine the potential effects of TQ on the dynamic thiol/disulphide homeostasis of rats that have received total-body IR, and to evaluate whether TQ was effective in terms of radioprotective activity.

MATERIAL AND METHODS
Study design and ethical guidelines

This study was conducted at the Necmettin Erbakan University KONUDAM Experimental Medicine Research and Study Center in Konya, Turkey. The animal protocol for this experiment was approved by the research and study centre's local ethics board for animal trials (No: 2011/073).

In vivo experiments and blood sampling

At the beginning of the experiment, 22 adult Wistar albino rats weighing 200–250 g were randomized into the following three treatment groups:

Group 1: IR group ($n = 8$): received only total-body IR of 6 Gy.
Group 2: TQ + IR group ($n = 8$): received IR (6 Gy) plus TQ (10 mg/kg, i.p., 30 min before IR).
Group 3: Sham control group ($n = 7$): did not receive TQ or IR.

Before the experiment, the rats were housed in wire cages at a temperature of 22°C and at 50–60% humidity, with 12 h night, 12 h day circadian rhythms. All procedures were conducted under sterile conditions. The rats were allowed access to sterile food. Twenty-four hours before the experiment, the rats were starved, but they

could drink water *ad libitum*. The rats in Group 2 were administered a dose of 10 mg/kg TQ (Sigma-Aldrich, St Louis, MO, USA) dissolved in dimethyl sulphoxide. Thirty minutes after the drug was administered intraperitoneally, all animals were anaesthetized with 50 mg/kg ketamine HCl. RT was applied under general anaesthesia to immobilize the rats prior to radiation exposure. The animals were placed on a plexiglass tray and stabilized in the supine position. Whole-body irradiation of rats was performed at 0.44 Gray (Gy) per minute, for a total dose of 6 Gy with 6 MV photon beams (Linear Accelerator, Siemens, PRIMUS). The source–axis distant technique was used, and the distance from source centre to skin was 100 cm. Each rat was exposed to a single dose of radiation. The animals were returned to their home cages following IR. One and a half hours after IR, anaesthesia was performed in the same way. Intracardiac blood was drawn into tubes containing ethylenediamine tetraacetic acid. The animals were euthanized under anaesthesia by exsanguination. The blood samples were centrifuged at 1500g for 10 min. The carefully separated plasma was kept at −80°C after coding.

Measurement of the dynamic thiol/disulphide homeostasis

Thiol/disulphide homeostasis tests were undertaken using a novel automatic and spectrophotometric method that measures the exact thiol/disulphide status. The principle of the thiol/disulphide measurement method is the reduction of dynamic disulphide bonds (–S–S–) to functional thiol groups (–SH) by $NaBH_4$. The unused $NaBH_4$ remnants are completely removed by formaldehyde. This prevents further reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as well as any disulphide bonds resulting from the reaction with DTNB. The modified Ellman reagent was used to quantify the total thiol content in the samples. The level of disulphide bonds is half of the difference between the native thiol content and the total thiol content. After the native and total thiols were determined, the disulphide levels, disulphide/total thiol percentage ratios, native thiol/total thiol percentage ratios and disulphide/native thiol percentage ratios were calculated [3].

Statistical analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Quantitative data were given as mean ± SD. Statistical comparison of the results was performed using the Kruskal–Wallis test and Dwass, Steel, Critchlow and Fligner (DSCF) multiple comparison analysis. A P value < 0.05 was considered statistically significant.

RESULTS

Data for a total of 22 rats, including 6 animals in the sham group and 8 animals in each of the other two groups, were evaluated in this study. The thiol/disulphide homeostasis parameters (native thiol, total thiol, disulphide levels and disulphide/total thiol, disulphide/native thiol and native thiol/total thiol ratios) of the groups are summarized in Table 1.

Among the three groups, native thiol levels were highest in the sham control group. The native thiol levels on the basis of IR and

Table 1. The thiol/disulphide homeostasis parameters of the groups

Parameter	Irradiation group ($n = 8$) Mean $\pm$ SD	Thymoquinone+irradiation group ($n = 8$) Mean $\pm$ SD	Sham control group ($n = 7$) Mean $\pm$ SD
Native thiol (SH) (μmol/l)	27.41 $\pm$ 6.13*	57.25 $\pm$ 51.02	71.31 $\pm$ 21.88
Total thiol (μmol/l)	111.3 $\pm$ 13.3*	159.6 $\pm$ 112.3	158.3 $\pm$ 24.9
Disulphide (SS) (μmol)	41.9 $\pm$ 6.9	51.2 $\pm$ 33.4	43.5 $\pm$ 5.15
Disulphide/native thiol (%)	164.3 $\pm$ 64.75*	133.3 $\pm$ 85.09	66.8 $\pm$ 22.84
Disulphide/total thiol (%)	37.57 $\pm$ 2.97*	33.68 $\pm$ 7.27	27.91 $\pm$ 4.09
Native thiol/total thiol (%)	24.84 $\pm$ 5.94*	32.62 $\pm$ 14.55	44.16 $\pm$ 8.19

*Significantly different when compared with sham control group ($P < 0.05$).
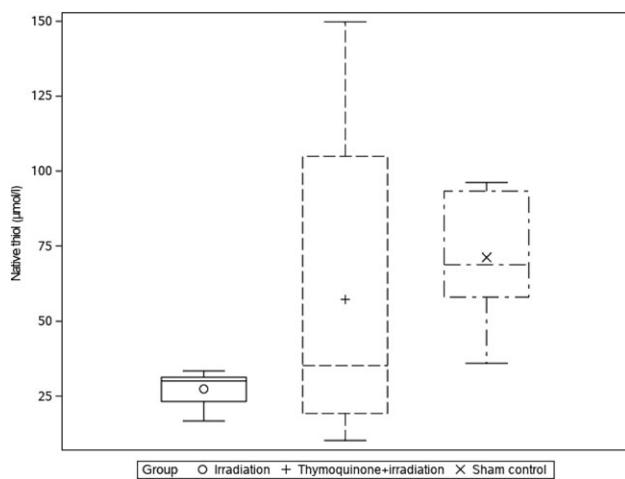
Fig. 1. Comparison of the native thiol levels between the groups.

sham control groups showed a considerable difference statistically ($P = 0.003$). The native thiol mean of the IR group was 27.41 $\pm$ 6.13 μmol/l, whereas the native thiol mean of the sham control group was 71.31 $\pm$ 21.88 μmol/l (Table 1).

The box plot graph for native thiol indicates that the IR group had the lowest levels of native thiol. Additionally, the range of the TQ + IR group was wider than that of the sham control group (Fig. 1).

Total thiol was significantly different between the sham control group and the two groups with IR ($P = 0.02$). The total thiol levels in the TQ + IR group were closer to those in the sham control group than to those (Table 1) (Fig. 2).

There was no statistically significant difference between the groups in terms of disulphide levels ($P > 0.05$). Mean disulphide levels were similar in all groups (Fig. 3).

The disulphide/native thiol ratio levels of the TQ + IR group were not found to differ from those of the other groups ($P > 0.05$). The mean disulphide/native thiol ratio of the subjects in the IR group was 164.3 $\pm$ 64.75, whereas it was 66.8 $\pm$ 22.84 in the sham control group (Table 1). The disulphide/native thiol ratio was least in the sham control group; only the disulphide/native thiol ratio of

the IR group was significantly higher than that of the sham control group ($P = 0.003$) (Fig. 4).

The disulphide/total thiol and native thiol/total thiol ratios were found to be significantly different between the sham control group and the two groups with IR ($P = 0.003$ and $P = 0.003$, respectively). There was no statistically significant difference between the sham control and the TQ + IR groups for these two rates ($P = 0.24$ for all), (Table 1), but the range of the TQ + IR group was wider than that of the sham control group in box plots (Fig. 5).

DISCUSSION

RT is the main pillar in cancer care, offering curative treatment for several indications. Despite the vast benefits derived from the various medical applications of RT, RT-related side effects sometimes develop [10]. Any agent that can decrease its toxicity to normal cells would therefore be of great value in reducing the very unpleasant side effects that cancer patients receiving RT can experience. In this study, the radioprotective effects of TQ in terms of thiol disulphide homeostasis were assessed in rats exposed to IR. Our results showed that in the TQ + IR group, the mean disulphide, native thiol and total thiol levels and disulphide/native thiol, disulphide/total thiol and native thiol/total thiol ratios were not found to be significantly different when compared with those of the sham control group. It was demonstrated that the combination of TQ with RT reduced RT-induced oxidative stress.

IR administered during RT generates free radicals when it passes through living tissues and generates reactive oxygen species (ROS) as a result of water radiolysis [11]. Although ROS play an important role in regulating cell proliferation and survival in small concentrations, they can induce cell death at higher levels [12]. ROS have very short half-lives and low concentrations, which makes their direct measurement very difficult in cells, tissues, or body fluids. Therefore, other indicators are used to quantify the status and potential of oxidative stress. These indicators include biomarkers that measure tissue destruction, e.g., lipid peroxidation, protein and DNA damage, and antioxidant concentration in blood [13].

Compounds containing sulphur in the form of thiol play a significant role in antioxidant cascades; they are important antioxidants that clean free radicals through enzymatic or non-enzymatic pathways [14]. Thiol metabolism and its role in oxidative stress have received considerable
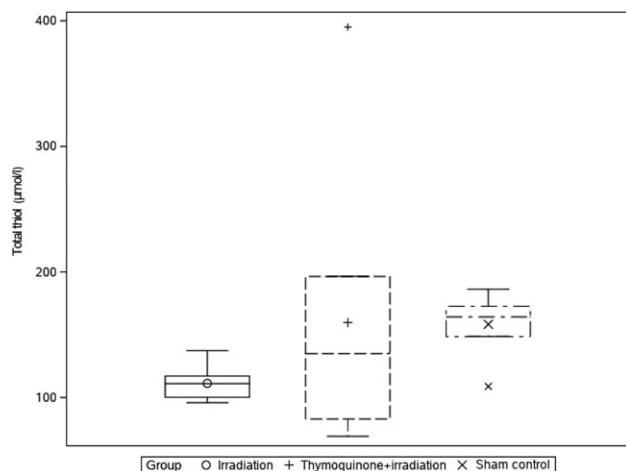
Fig. 2. Comparison of the total thiol levels between the groups.
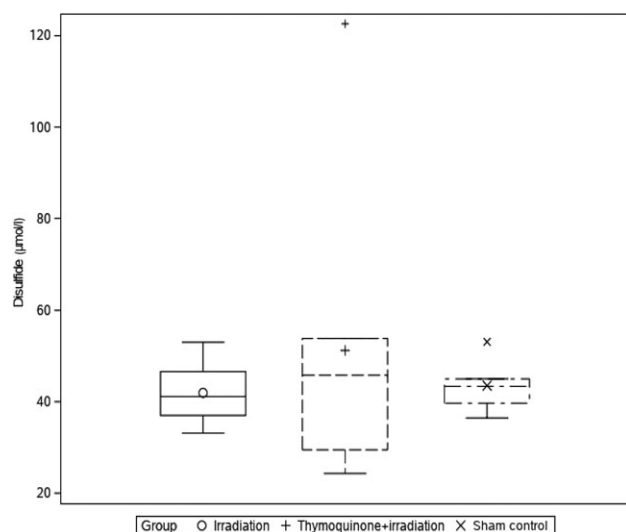

Fig. 4. Comparison of the disulphide/native thiol ratios between the groups.

Fig. 3. Comparison of the disulphide levels between the groups.

recent attention in comparison with that given to the widely used classical biomarkers of oxidative stress [15]. Plasma thiol pools are primarily formed by protein thiols, albumin thiols and thiols with a lower degree and molecular weight. These organic compounds contain a sulphydryl group and play a critical role in preventing oxidative stress. Thiol groups containing amino acids such as cysteine and methionine in a protein structure are the primary targets of ROS. Thiol groups form a disulphide bond with ROS through oxidation. The thiol levels in the plasma may provide an indirect assessment of oxidative damage. Thiol groups reversibly turning into disulphide structures may cause a decrease in thiol groups. Thus, thiol/disulphide homeostasis is preserved. Thiol/disulphide homeostasis plays a significant role in detoxification, signal transfer, enzyme activity organization and apoptosis [14, 16].

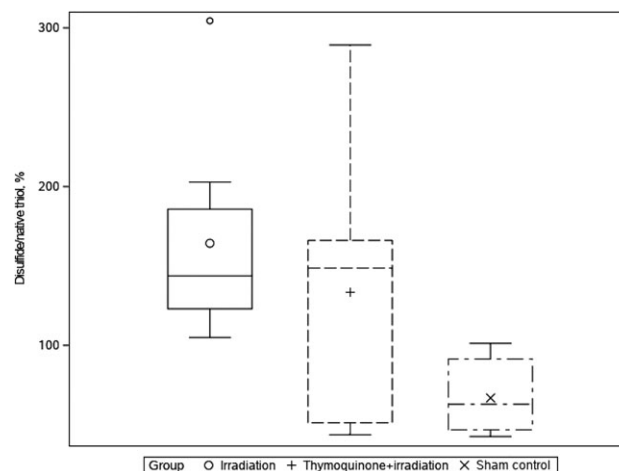The classical Ellman method of analysis enables determination of total thiol levels, and previous studies were based on this method [17]. The development of a new method by Erel and Neselioglu has made it possible to measure low-molecular-weight native thiols, disulphides and total thiol levels *in vivo* [3]. To the best of our knowledge, this is the first study investigating thiol/disulphide homeostasis as a novel oxidative stress marker using this newly defined method in rats that have received total-body IR and TQ. Native thiol levels were found to be lower in the IR-exposed group, which was consistent with previous findings that IR induces oxidative stress [11, 18]. We also found that disulphide/native thiol and disulphide/total thiol ratios, which are formed by thiol oxidation, were higher in IR-exposed subjects. Biaglow *et al.* reported that protein thiol status alterations are a critical factor in cell survival after IR, and that they can be demonstrated *in vitro* under controlled conditions [19].

Antioxidant activity is considered to be one of the major mechanisms by which many plants and phytochemicals are reported to offer radioprotection [20]. TQ was found to have antioxidant effects in animal models [21, 22]. Cemek *et al.* demonstrated that TQ significantly reduced blood oxidative stress markers such as malondialdehyde, nitrate, nitrite, glutathione and ceruloplasmin in rats exposed to a single dose of 6 Gy radiation. These antioxidant effects were also thought to protect tissues from radiation injury [23]. Akyuz *et al.* investigated the radioprotective effects of TQ against radiation-induced damage in the salivary glands of rats. They found that by reducing the formation of nitric oxide, peroxynitrite, and malondialdehyde, and decreasing xanthine oxidase and nitric oxide synthase activities, TQ had antioxidant effects and free radical scavenging activity [24]. Although these studies demonstrate the ability of TQ to reduce oxidative stress using oxidant and antioxidant parameters, none of them evaluated the effect of TQ on thiol/disulphide homeostasis. According to our study, oxidative stress accompanied by damaged thiol/disulphide homeostasis was present in rats that received IR, and TQ treatment significantly reduced the adverse effects of IR. Additionally, the new spectrophotometric method used led us to measure oxidative status more precisely.
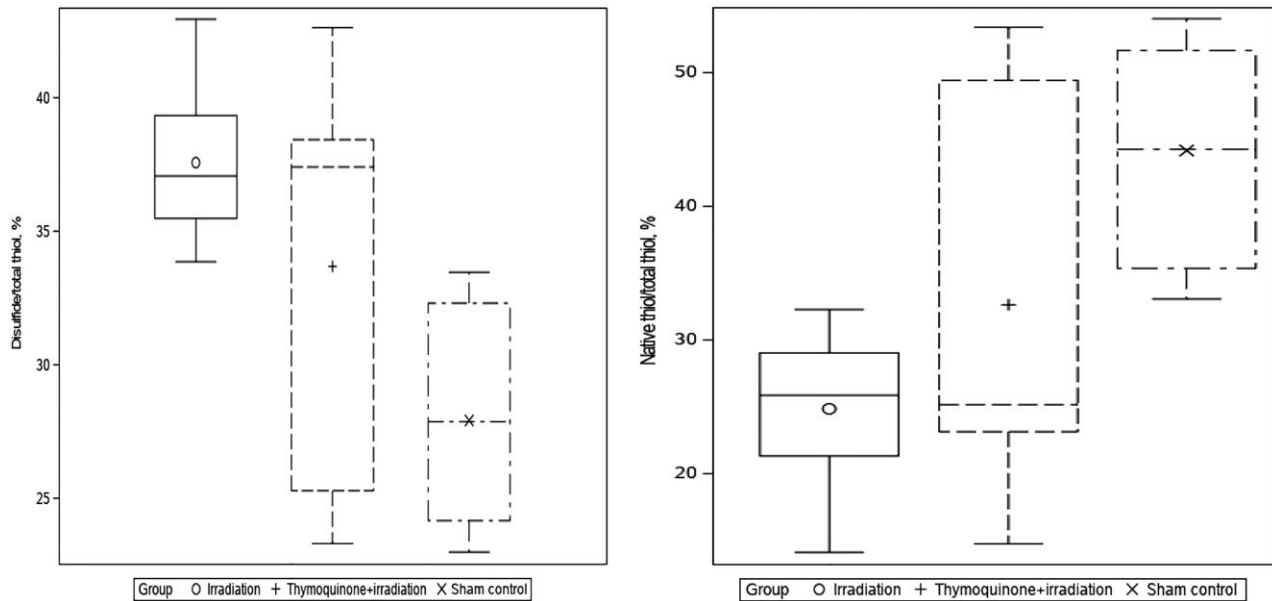
Figure 5. Comparison of the disulphide/total thiol and native thiol/total thiol ratios between the groups.

Traditional medicine is a promising source of new therapeutics against cancer. As such, there is increasing interest in natural products for complementing conventional medicine. We demonstrated beneficial effects of TQ on thiol/disulphide homeostasis when administered before RT. Therefore, we hypothesize that the use of TQ before radiation treatment may help to protect against IR-related oxidant side effects. Extensive research into TQ may contribute to the discovery of new anticancer strategies.

In conclusion, thiol/disulphide homeostasis was found to be disturbed after IR exposure. Our results showed that TQ has antioxidant effects and reduces IR-induced oxidative stress, which can be demonstrated through dynamic thiol/disulphide homeostasis. This is the first study that highlights an association between thiol/disulphide homeostasis and TQ during total-body IR. The novel spectrophotometric method used in this study helped provide more accurate and precise measurements of oxidative status.

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