MITIGATION OF THE GENOMIC INSTABILITY CAUSED BY IRON INDUCED FREE RADICALS THROUGH SOME ANTIOXIDANTS IN VIVO

ABSTRACT
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Iron is by far the most abundant transition metal in the human body. It plays vital roles in oxygen binding and transport and electron transport. The human body contains approximately 3-5 g of iron (45-55 mg/kg of body weight) in adult women and men, respectively. Iron has central and essential roles in the metabolism of all aerobic organisms. Iron catalyzes production of hydroxyl radicals which intensify oxidative stress (Parveen and Shadab, 2012). Several studies have been carried out to address the problem of potential toxicity induced by iron and also by drugs and compounds containing this metal. Humans have evolved some peculiar ways of dealing with it. These peculiarities provide opportunities for the cause of many diseases related to iron absorption, transport, and metabolism, as well as for the exacerbation of general mechanisms of disease involving free radical injury (McCord, 2004).

Iron loading or iron mismanagement has now been recognized as a serious risk factor associated with a considerable profusion of diseases like amyotrophic lateral sclerosis, breast cancer, colorectal cancer, hepatic carcinoma, depression, Down syndrome, epilepsy, hypertension, inflammatory bowel disease, ischemic stroke, leukemia, pre-eclampsia, venous leg ulcer, porphyria cutanea tarda, sudden infant death syndrome etc. (Weinberg and Miklossy, 2008). The amount of iron within the cell has to be carefully regulated in order to provide an adequate level of the micronutrient while preventing its accumulation to toxic levels. Mitigation of such harmful effects has drawn attention of researchers globally specially by using natural products including thymoquinone (TQ) and quercetin (QCT), which are supposed to possess strong antioxidant activity and protective roles.

TQ (2-methyl-5-isopropyl-1,4-benzoquinone), is the most bioactive compound isolated from *Nigella sativa*. Investigations for its antioxidant, anti-inflammatory and anticancer activities in both *in vitro* and *in vivo* models have shown promising results. TQ was extracted by Gad *et al.* (1963), and in numbers of studies this compound has been evaluated for its pharmaceutical and therapeutic effects in many diseases including inflammation, cancer, sepsis, atherosclerosis and diabetes (Woo *et al.*, 2012). Mansour *et al.* (2002) reported that TQ has very strong free radical and superoxide radical scavenger activity and shows anti-inflammatory activity in animal models and cell culture systems.
at both nanomolar and micromolar range, respectively. This was consistent with the report by Badary et al. (2003) showing that TQ is a potent superoxide anion scavenger of various reactive oxygen species such as superoxide anion radicals and hydroxyl radicals. It has been shown to efficiently inhibit iron-dependent microsomal lipid peroxidation in rats with doxorubicin induced hyperlipidemic nephropathy (Badary et al., 2000). These findings suggest that TQ is a strong radical scavenger with a potential role in the prevention and treatment of oxidative stress related pathogenesis.

Flavonoids are also best known for their antioxidant properties, and may act in vitro as reducing agents, hydrogen donors, free radical quenchers and metal ion chelators (Shahidi, 1992). QCT (3, 5, 7, 3', 4'-pentahydroxy-flavone) is one of the most commonly occurring flavonoids and is ingested with edible fruits and vegetables at levels of up to 16 mg per day (Hertog et al., 1993). It has been shown to possess anticarcinogenic abilities, which are attributable to its anti-oxidative capacity (Van Acker et al., 1996) or to other mechanisms of anticarcinogenicity in animal studies (Stavric, 1994). The studies described above suggest strong effect of QCT and provide insight into the mechanism of the antioxidant effects. Evidence of efficacy in in vivo studies is more limited.

Many naturally occurring compounds have been reported to have antigenotoxic and cancer-preventive effects. Many plant-derived foods, fruits, vegetables, herbs and spices and their isolated phytochemicals and flavonoids have been claimed to have antimutagenic and anticarcinogenic activities. A systematic screening of plant extracts, food supplements or dietary products for their potential chemopreventive and antimutagenic activities against chemical carcinogens is urgently needed. There is a lack of knowledge about the mechanism underlying suggested beneficial health effects instigated by consumption of fruits and vegetables, where phytochemicals and flavonoids are thought to play an important role. As described earlier, damage to DNA can lead to several diseases including cancer; therefore, protection against DNA damage can be regarded as an ultimate prevention of the disease. Intake of fruits and vegetables has been suggested as a way of defense against DNA damage and thus, against degenerative diseases. In order to attain insight in this phenomenon, a rat model situation would be valuable because of their availability and accessibility.
In this thesis first part covers an insight into effectiveness of iron inducing chromosomal aberrations, micronucleus and DNA damage.

And second part was done to obtain more precise data of mitigation by TQ and to obtain its optimum level at which it exhibits maximum protection against genotoxicity induced by iron.

Third part was undertaken to have insight into effectiveness of QCT in ameliorating genotoxicity induced by iron and to obtain optimum level of QCT at which it shows maximum protection.

In this approach we tried to confirm anti-oxidative efficacy of TQ and QCT. This thesis sets out to collect evidence for ameliorating effects of chosen natural antioxidants that would be useful to minimize the genotoxic effects of iron induced free radicals and awareness about the choice and dose of antioxidants along with iron drugs to mitigate the genotoxic effects during treatment.

Therefore the objectives of the present work are:

- To study the genotoxic effects of iron induced free radicals on cytogenetic and molecular parameters \textit{in vivo} by using chromosomal aberrations, micronucleus and comet assay.
- To investigate the ameliorative effect of thymoquinone pre, simultaneous and post treatment and its optimum level against genotoxicity induced by iron by using chromosomal aberrations, micronucleus and comet assay in Wistar rats.
- To investigate the ameliorative effect of quercetin pre, simultaneous and post treatment and its optimum level against genotoxicity induced by iron by using chromosomal aberrations, micronucleus and comet assay in Wistar rats.

The number of animals used for each group was enough to lessen the effects of animal to animal variations in response, and also to enable the required number of cells to be obtained without analyzing a large number from any one animal. In the present study rats were divided in 23 groups of 6 animals each. The experiment was divided into three parts. The first part was done to determine the maximum genotoxic dose of iron sulfate among the doses of 50, 100 and 200 mgFe/kg on chromosomal aberration, micronucleus and single cell gel electrophoresis (SCGE/Comet assay) in bone marrow and whole
Abstract

Blood cells of rats. The rats were separated randomly into 5 groups of 6 animals each. The first group was used as negative control and administered DMSO and second group was used as positive control and administered 25 mg/kg cyclophosphamide i.p. Groups 3-5 were administered FeSO₄ at the doses of 50, 100 and 200 mg Fe/kg p.o. The second part was done to observe the optimum level of TQ on chromosome aberrations, micronucleus and DNA damage induced by FeSO₄ 200 mg Fe/kg. The rats were separated randomly into 9 groups of 6 animals each. Group 1 was used as negative control and administered DMSO, group 2 was administered FeSO₄ at the doses of 200 mg Fe/kg and group 3-9 were administered pre, simultaneous and post treatment of TQ at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg with the administration of FeSO₄ 200 mg Fe/kg. The third part was done to observe the optimum level of QT on chromosome aberrations, micronucleus and DNA damage induced by FeSO₄ 200 mg Fe/kg. The rats were separated randomly into 9 groups of 6 animals each. Group 1 was used as negative control and administered with DMSO and Group 2 was administered FeSO₄ at the dose of 200 mg Fe/kg and groups 3-9 were administered pre, simultaneous and post treatment of QT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg with the administration of FeSO₄ 200 mg Fe/kg.

Bone marrow cells were obtained for chromosomal aberration assay from the rats using the technique described by Preston et al. (1987). Bone marrow preparation was made for micronucleus test according to Schmid (1975). Single cell gel electrophoresis (SCGE/Comet assay) was performed in dark according to the method described by Buschini et al. (2002) with slight modifications. Data are expressed as the mean ± SD and were analyzed using one-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples with the help of SPSS (version 16). The level of significance was set at P <0.05. Broken-line regression analysis was employed to determine the optimum level of TQ (Robbins et al., 1979). The equation employed was Y= a+bX. Statistical analysis was done using Origin (version 6.1; Origin Software, San Clemente, CA, USA).

Structural aberrations (chromatid and chromosome types) induced by different treatments have been enumerated in the present study, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings. All the three doses (50, 100 and 200 mgFe/kg) of iron sulfate induced dose dependent
increase in the mean number of aberrant cells at 24 h of treatment when compared to the negative control. Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the total number of structural chromosome aberrations when compared with the negative control ($P < 0.001$). Statistical analysis showed that there were also significant differences in the total number of structural chromosome aberrations between the control and cells treated with iron sulfate and TQ in the pre, simultaneous and post-treatments (for all the treatments $P < 0.001$). In the presence of TQ (6, 9, 12, 15 and 18 mg/kg), the number of chromosomal aberrations statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P < 0.001$ (except at 21 and 24 mg/kg doses of TQ). Statistical analysis showed that there were also significant differences in the total number of structural chromosome aberrations between the negative control and cells treated with iron sulfate and QCT in the pre, simultaneous and post-treatments (for all the treatments $P < 0.001$). In the presence of QCT (125, 250, 375 and 500 mg/kg), the number of chromosomal aberrations statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P < 0.001$ (except at 625, 750 and 875 mg/kg doses of QCT). The ameliorating effect of TQ and QCT on iron sulfate induced chromosome aberrations was most prominent in simultaneous treatments.

The number of MNPCEs among 2000 PCE, indicative of genotoxicity. No statistically significant increase in the mean number of MNPCEs was observed in animals treated with iron sulfate at the dose of 50 and 100 mgFe/kg in comparison to the negative control. A statistically significant ($P<0.001$) increase in the mean number of MNPCEs was observed in the animals treated with 200 mgFe/kg compared to the negative control group. TQ at the doses of 6, 9, 12, 15 and 18 mg/kg, induced statistically significant decrease in the yields of MN induced by iron sulfate in pre, simultaneous and post treatments. TQ showed significant protective effect on iron sulfate induced MN in PCE. All doses of TQ tested were found to be effective in reducing the frequency of MN induced by iron sulfate except two highest doses (21 and 24 mg/kg doses of TQ). The most effectively significant inhibitory effect on MN in PCE ($P < 0.001$) was observed at 18 mg/kg dose of TQ. On the other hand QCT at the doses of 125, 250, 375 and 500 mg/kg, induced statistically significant decreases in the yields of MN induced by iron sulfate in pre, simultaneous and post treatments. QCT showed significant protective
Abstract

Iron overdose is one of the most common causes of poisoning deaths in children under 6 years of age in the United State. Poisoning symptoms occur with doses above 20 to 60 mg/kg of iron, with the low end of the range associated primarily with gastrointestinal irritation while systemic toxicity occurs at the high end (IOM, 2001)) and dose between...
10 and 20 mg Fe/kg are regarded as non toxic to humans (Schumann, 2001). Iron sulfate is a common chemical that is present in foods and beverages and that is used to treat iron deficiency anemia (Nelson and Cox, 2002). Despite this, in humans, several health problems have been related to high Fe intake (Chau et al., 1993), and as found in this study, it can also cause genotoxic damage.

The protective action of TQ and QCT observed here suggests that it has antioxidative property and damage induced by free radicals generated during the metabolic activity of iron sulfate in bone marrow and blood cells of rats was significantly ameliorated. TQ and QCT have been found more effective in protection against iron-induced free radicals via increased resistance to oxidative stress as well as the ability to reduce ROS production. Based on the broken line regression analysis of chromosomal aberrations, micronucleus and DNA damage data; it appears that the optimum levels of TQ and QCT at which they show their maximum protective effects against genotoxicity induced by iron sulfate are 18 mg/kg and 500 mg/kg.

Results of bone marrow chromosomal aberration assay, micronucleus assay and single cell gel electrophoretic assay (Comet assay), showed that TQ at 18 mg/kg and QCT at 500 mg/kg dose significantly decreased chromosomal aberrations, yields of micronuclei and DNA damage caused by iron sulfate. The study provides evidence that TQ and QCT inhibit in vivo genotoxicity of iron sulfate in rats. Meanwhile, efforts should continue focusing laboratory research to gain further in-depth understanding of the antioxidant property of these nature endowed compounds for therapeutic uses in humans.