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

ABSTRACT OF THE THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE OF
Doctor of Philosophy
IN
ZOOLOGY (Genetics)

BY
NUZHAT PARVEEN

SECTION OF GENETICS
DEPARTMENT OF ZOOLOGY
ALIGARH MUSLIM UNIVERSITY
ALIGARH-202002 (U.P.) INDIA

2013
THESIS

ABSTRACT

THE PROBLEM

DESCRIPTIVE

METHODOLOGY

RESULTS

DISCUSSION

REFERENCES

ANNEXURES
ABSTRACT

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.
Abstract

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/-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 antimitogenic and anticarcinogenic activities. A systematic screening of plant extracts, food supplements or dietary products for their potential chemopreventive and antimitogenic 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 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
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
effect on iron sulfate induced MN in PCE. Among all the doses of QCT tested were found to be effective in reducing the frequency of MN induced by iron sulfate (except at 625, 750 and 875 mg/kg doses of QCT). The most effectively significant inhibitory effect of QCT on MN in PCE ($P < 0.001$) was observed at 500 mg/kg dose of QCT. The protective influence of TQ and QCT on iron sulfate induced number of micronuclei was most prominent in the simultaneous treatments.

The effects of the iron sulfate and TQ pre, simultaneous and post treatment on the extent of DNA damage as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. Treatment by iron sulfate at the dose of 50 and 100 mgFe/kg did not induce any significant increase in mean tail moment and olive tail moment. However, significantly higher levels of DNA damage were detected in rats exposed to iron sulfate at the dose of 200 mgFe/kg when compared with the negative control. Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a concentration of 200 mg/kg compared with the negative control ($P<0.001$). Statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the negative control and from animals treated with iron sulfate and TQ in the pre, simultaneous and post-treatments, $P<0.001$ (except 21 and 24 mg/kg doses of TQ). In the presence of TQ doses (6, 9, 12, 15 and 18 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P < 0.001$. In case of QCT also statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the negative control and cells treated with iron sulfate and QCT in the pre, simultaneous and post-treatments (except 625, 750 and 875 mg/kg dose of QCT $P<0.001$). In the presence of QCT doses (125, 250, 375 and 500 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P<0.001$. The protective influence of QCT on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in 500 mg/kg. The protective influence of TQ and QCT on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in simultaneous treatments.

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

THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE OF
Doctor of Philosophy
IN
ZOOLOGY
(Genetics)

NUZHAT PARVEEN

SECTION OF GENETICS
DEPARTMENT OF ZOOLOGY
ALIGARH MUSLIM UNIVERSITY
ALIGARH-202002 (U.P.) INDIA

2013
First teacher I have in my life are my parents.

Dedicated to my loving parents

Words cannot express my deepest gratitude to my parents for their love, patience, support and sacrifices, which is vested on every page of this study and is pivot in the completion of this task.
Certificate

I feel great pleasure in forwarding the thesis entitled "Mitigation of the Genomic Instability Caused by Iron Induced Free Radicals through Some Antioxidants in vivo" embodies the original research work carried out by Ms. Nuzhat Parveen, Research Scholar, Department of Zoology for the award of the degree of Doctor of Philosophy (Zoology, Genetics) of the Aligarh Muslim University, Aligarh. This is to certify that the subject matter of the thesis as presented by Ms. Parveen is the actual record of work done under my supervision and guidance and the same does not form the part of any other course or degree presented to or taken by her.

Dr. G. G. H. A. Shadab
(Supervisor)
CONTENTS

Acknowledgements i-ii
List of Tables iii-iv
List of Figures v-ix

1. INTRODUCTION 1-38

1.1. GENOMIC INSTABILITY 4-5

1.2. IRON 5

1.2.1. Iron – the element 5

1.2.2. Iron – the nutrient 6

1.2.3. Iron Chemistry 7

1.2.4. Body Iron and Its Physiological Role 7-8

1.2.5. Body Iron Homeostasis 8-9

1.2.6. Iron Absorption and Distribution in Humans 9

1.3. TOXICITY OF IRON 9-15

1.3.1. Iron Induced Free Radicals Which Leads to 9-12

Oxidative Stress

1.3.2. Iron must be handled very carefully due to its ability to catalyze 12-13

potentially destructive redox chemistry

1.3.3. Genotoxicity of Iron 13-15

1.3.4. The Roles of Iron in Health and Diseases 15

1.4. SOME CONDITIONS THAT COMPROMISE THE 15-16

IRON WITHHOLDING DEFENSE SYSTEM

1.4.1. Genetic disorders 15

1.4.2. Behavioral factors 15

1.4.3. Pathological conditions 16

1.5. DISEASES FOR WHICH EXCESSIVE/MISPLACED 17-19

IRON CAN BE A RISK FACTOR

Examples of action of iron in specific diseases

1.5.1. Iron, by itself can initiate the disease 18

1.5.2. Iron can be a cofactor in promoting the disease 18
1.5.3. Iron deposits are observed in disease-associated tissue sites

1.5.4. Body iron loading is associated with above-normal incidence of disease

1.5.5. Maternal antibodies can impair fetal iron metabolism

1.5.6. Iron Induced Carcinogenesis in Humans and Animals

1.6. EFFECT OF NATURAL ANTI OXIDANT ON FREE RADICALS PRODUCTION AND REMOVAL

1.6.1. ANTIOXIDANT POTENTIAL OF THYMOQUINONE

1.6.1.1. Antioxidant Activity

1.6.1.2. Thymoquinone as a free radical scavenger

1.6.1.3. Anti-Inflammatory and Chemopreventive Activity of TQ

1.6.1.4. Antiproliferative Activity of TQ

1.6.1.5. Cell Cycle Regulation by TQ

1.6.1.6. TQ and Apoptosis

1.6.1.7. TQ Inhibits Angiogenesis and Endothelial Cell Functions

1.6.1.8. In Vivo Antitumor Activity

1.6.1.9. Therapeutic Uses

1.6.2. ANTIOXIDANT POTENTIAL OF QUERCETIN

1.6.2.1. Properties, Structure, Production and Occurrence

1.6.2.2. Biosynthesis of Quercetin

1.6.2.3. Absorption, Metabolism, Distribution and Excretion

1.6.2.4. Mechanism of Action

1.6.2.4.1. Anti-oxidative action

1.6.2.4.2. Direct radical scavenging action

1.6.2.4.3. Inducible nitric oxide synthesis inhibitory action

1.6.2.4.4. Xanthine oxidase inhibitory action

1.6.2.5. Therapeutic Uses

2. OBJECTIVES

3. MATERIALS AND METHODS

3.1. TEST ANIMALS

3.1.1. Species And Strains
3.1.2. Sex 41
3.1.3. Weight 41
3.1.4. Age 41
3.1.5. Housing 41-42
3.1.6. Chemicals 42

3.2. CHEMICAL TREATMENTS 42-44
3.2.1. Route of Administration 42
3.2.2. Solvent or Vehicle 42
3.2.3. Dose Selection 43
3.2.4. Negative Controls 43
3.2.5. Positive Control 43
3.2.6. Number of Animals and Administration of doses 43-44

3.3. MAMMALIAN BONE MARROW CHROMOSOMAL ABERRATION ASSAY 44-46
3.3.1. PRINCIPLE 44-45
3.3.2. SAMPLING TIMES AND SLIDE PREPARATION 45-46
  3.3.2.1. Sampling Time 45
  3.3.2.2. Slide Preparation, Sacrifice and Harvest 45
  3.3.2.3. Hypotonic Treatment 46
  3.3.2.4. Fixation 46
  3.3.2.5. Slide Preparation 46
  3.3.2.6. Scoring and Photography 46

3.4. MICRONUCLEUS ASSAY 47-51
3.4.1. PRINCIPLE 47-49
3.4.2. SAMPLING TIMES AND SLIDE PREPARATION 49-51
  3.4.2.1. Sampling time 49
  3.4.2.2. Extraction of bone marrow from animals 49-50
  3.4.2.3. Preparation of the smears 50
  3.4.2.4. Staining 50
  3.4.2.5. Analyzing the slides 50-51

3.5. SINGLE CELL GEL ELECTROPHORESIS 51-53 (SCGE/COMET ASSAY)
3.5.1. PRINCIPLE 51-52
3.5.2. SAMPLING TIMES AND SLIDE PREPARATION 52-53
  3.5.2.1. Sampling time 52
3.5.2.2. Blood collection 52
3.5.2.3. Slide preparation 52-53
3.5.2.4. Lysis 53
3.5.2.5. Unwinding and electrophoresis 53
3.5.2.6. Neutralization and dehydration of slides 53
3.5.2.7. DNA staining, comet visualization and analysis 53

4. RESULTS 54-114

4.1. ACUTE TOXICITY OF IRON SULFATE 54-62

4.1.1. Evaluation of Acute Toxicity of Iron Sulfate by Chromosomal Aberration Assay in Bone Marrow Cells of Wistar rats 54-56

4.1.2. Assessment of Acute Toxicity of Iron Sulfate by Micronucleus Assay in Bone Marrow Cells 57-59

4.1.3. Assessment of Acute Toxicity of Iron Sulfate by single cell gel electrophoresis (SCGE/Comet Assay) 60-62

4.2. PROTECTIVE EFFECTS OF THYMOQUINONE 63-88

4.2.1. Protective Effect of Thymoquinone on iron sulfate induced Chromosome Aberrations 63-71

4.2.1.1. Pre-Treatment with Thymoquinone

4.2.1.2. Optimum level of TQ after Pre-treatment

4.2.1.3. Simultaneous Treatment with Thymoquinone

4.2.1.4. Optimum level of TQ after Simultaneous treatment

4.2.1.5. Post Treatment with Thymoquinone

4.2.1.6. Optimum level of TQ after Post-treatment

4.2.2. Protective Effects of Thymoquinone on Micronucleus Induced By Iron Sulfate 72-76

4.2.2.1. Pre-Treatment with Thymoquinone

4.2.2.2. Optimum level of TQ after Pre-treatment

4.2.2.3. Simultaneous Treatment with Thymoquinone
4.2.2.4. Optimum level of TQ after Simultaneous treatment

4.2.2.5. Post Treatment with Thymoquinone

4.2.2.6. Optimum level of TQ after Post-treatment

4.2.3. Protective Effect of Thymoquinone on DNA Damage Induced By Iron Sulfate  

4.2.3.1. Pre-Treatment with Thymoquinone

4.2.3.2. Optimum level of TQ after Pre-treatment

4.2.3.3. Simultaneous Treatment with Thymoquinone

4.2.3.4. Optimum level of TQ after Simultaneous treatment

4.2.3.5. Post Treatment with Thymoquinone

4.2.3.6. Optimum level of TQ after Post-treatment

4.3. PROTECTIVE EFFECTS OF QUERCETIN  

4.3.1. Protective Effects of Quercetin on Chromosome Aberrations Induced By Iron Sulfate  

4.3.1.1. Pre-Treatment with Quercetin

4.3.1.2. Optimum level of QCT after Pre-treatment

4.3.1.3. Simultaneous Treatment with Quercetin

4.3.1.4. Optimum level of QCT after Simultaneous treatment

4.3.1.5. Post Treatment with Thymoquinone

4.3.1.6. Optimum level of QCT after Post-treatment

4.3.2. Protective Effects of Quercetin on Micronucleus Induced By Iron Sulfate  

4.3.2.1. Pre-Treatment with Quercetin

4.3.2.2. Optimum level of QCT after Pre-treatment

4.3.2.3. Simultaneous Treatment with Quercetin

4.3.2.4. Optimum level of QCT after Simultaneous treatment

4.3.2.5. Post Treatment with Quercetin

4.3.2.6. Optimum level of QCT after Post-treatment
4.3.3. Protective Effect of Quercetin on DNA Damage Induced By Iron Sulfate 103-114

4.3.3.1. Pre-Treatment with Quercetin
4.3.3.2. Optimum level of QCT after Pre-treatment
4.3.3.3. Simultaneous Treatment with Quercetin
4.3.3.4. Optimum level of QCT after Simultaneous treatment
4.3.3.5. Post Treatment with Quercetin
4.3.3.6. Optimum level of QCT after Post-treatment

5. DISCUSSION 115-126
6. BIBLIOGRAPHY 127-155
   APPENDICES 156-159
   ABBREVIATIONS 156
   REAGENT PREPARATIONS 157-159
ACKNOWLEDGEMENTS

This thesis is the end of my journey in obtaining my Ph.D. During the entire course of my investigation, starting from its inception and finally the crystallization of the results, there has been a constant source of encouragement of numerous people including my well-wishers, my friends, colleagues and various institutions.

I accredit my sincere thanks to The Chairman, Department of Zoology for providing necessary infrastructure, resources and constant help which he bestowed upon me to demystify the hurdles falling on my way to accomplish my research work.

I am thankful to my supervisor Dr. G.G.M.A. Shadab for scholarly advice, constant encouragement, invaluable comments and discussion in every phase of my research career. This thesis is largely a result of his diligent and meticulous approach in each and every step of work.

I tender my immense indebtedness and obligations to all my esteemed teachers, Prof. Irfan Ahmad, Prof. Iqbal Parwez, Prof. Waseem Ahmad Faridi, Prof. Asif Ali Khan, Prof. Wasim Ahmad, Prof. Mohammad Afzal, Prof. Wajibullah, Prof. Mukhtar Ahmad Khan, Prof. Qudsia Tahseen, Prof. S.M.A. Abidi, Dr. M. K. Ahmad, Kamil Usmani, Dr. M. Afzal Khan, Dr. Shahid Bin Zeya, Dr. M. Khadija Saifullah, Dr. S. M. Abbas Abidi, Dr. Malik Irshadullah, Dr. Riaz Ahmad and Dr. Yasin Hassan Siddique for their help and cooperation in all possible ways to make this work a success. This list is incomplete without acknowledging Dr. Kamal Kumar Saxena, Dr. Mahesh Verma, and Dr. Javed Abdul Wejida, who were the source of inspiration for me in my early days and who taught me many things.

The road to my Ph.D. started with training at NBFCR, Lucknow. I take this opportunity to say heartfelt thanks to Dr. Nagpure and Dr. Kushwaha for providing me experimental Hands-On Training on Current Tools for Genotoxicity Assessment and building confidence in me and expertise in chromosome alteration, micronucleus as well as in comet assay techniques.

Most of the results described in this thesis would not have been obtained without a close collaboration with few laboratories. I owe a great deal of appreciation and gratitude to Dr. Anup Kumar Tewari, Department of Parasitology and Dr. A. G. Telang, Division of Pharmacology and Toxicology, IVRI, Bareilly and Dr. Sarika Singh, CDRJ, Lucknow for providing me instrumentation facilities to carry out part of my research work at their institutes. My warm appreciation goes to all the research students of those labs.
I convey special acknowledgement to my trainee friends and respected teachers, Dr. Vijayta Triwari (IVRI), Ramjaneyulu (Iramal Life Sciences Limited, Mumbai), Dr. Anurag (NBPGR, Lucknow), Muheeb Beg (CDRI, Lucknow), Krishna Kumar (MGDC, Tamilnadu), who were always there when I really needed. Their enthusiasm and heart-warming welcome will always remain fresh in my memory.

I would also like to thank some people from early days of my research tenure. Dr. Hifzur Rehman Siddique, Dr. Mumtaz Jabeen, Dr. Fauzia Khan and Dr. Asma Farhat Sherwani were among those who kept me going at the beginning. It's my fortune to gratefully acknowledge to Dr. Md. Mahmood Khan and Subuki Ahidi, who helped me a lot. I, specially, would like to thank Dr. Sheeb Ahmad for their incredible help and assistance.

I am indebted to my many student colleagues for providing a stimulating and fun filled environment. I am thankful to Areeba Ahmad, Nazia Nazam, Monica Sharma, Pakiha Firdous, Raviya Fatima, Mohammad Aatif, Mahno Fatima, Falak Naz, Nisha Sharma, Uzma Tauheed, Uzma Rafi and Yusra Zaidi. My special appreciation goes to Dr. Habeeba and Ms. Sumebon for their friendship and encouragement. I wish to thank my best friends, Pooja, Suraiya and Smita for their love, care and moral support.

I would also like to extend huge, warm thanks to my roommate, Kusumika Sarkar for her support, generous care and the homely feeling at hostel. She was always beside me during the happy and hard moments to push me and motivate me.

It is indispensable for me to express my heartfelt gratitude, which goes to my hostel friends, Aisha, Nazish, Nasem-Ur-Saba, Shehla, Shagma, Sana, Farheen, Nilofar, Nida, Farah, Gazala, Afsha Aapa, Gazala Aapa, Zeenat Aapa,. Thank you doesn't seem sufficient but it is said with appreciation and respect to all of them for their support, encouragement, care, understanding and precious friendship.

I must express my thanks to the staff of the Department and Hostel for their cooperation and assistance.

Words cannot express my deepest gratitude to my parents for their love, patience, support and sacrifices, which is vested on every page of this study and is pivot in the completion of this task. They always stood behind me during all sweet and sour moments of my life. Whenever I get tired and desperate, I found them ready to provide me courage and consolation. Finally, I cannot leave out my sister, Ms. Uzma and brothers, Dr. Moazzam and Shafinawaz who have always been there for me with their prayers and advice.

I am grateful from the core of my heart to all those persons who knowingly and unknowingly helped me in the completion of this work.

[Signature]

e: Nazmat Parveen
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Titles</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chromosomal aberrations in Wistar rat bone marrow cells treated with iron sulfate and respective control for 24 h.</td>
<td>55</td>
</tr>
<tr>
<td>2.</td>
<td>Iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats for 24 h.</td>
<td>58</td>
</tr>
<tr>
<td>3.</td>
<td>Mean and standard deviation of comet tail moment and olive tail moment in whole blood of Wistar rats treated with iron sulfate for 24 h.</td>
<td>61</td>
</tr>
<tr>
<td>4.</td>
<td>The apportionment of structural aberrations observed in bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of TQ pre-treatment.</td>
<td>64</td>
</tr>
<tr>
<td>5.</td>
<td>The apportionment of structural aberrations observed in bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of TQ simultaneous treatment.</td>
<td>67</td>
</tr>
<tr>
<td>6.</td>
<td>The apportionment of structural aberrations observed in bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of TQ post treatment.</td>
<td>70</td>
</tr>
<tr>
<td>7.</td>
<td>Pre, simultaneous and post treatment of TQ - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.</td>
<td>75</td>
</tr>
<tr>
<td>8.</td>
<td>Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone and pre-treatment in combination with different doses of TQ for 24 h.</td>
<td>79</td>
</tr>
<tr>
<td>9.</td>
<td>Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone and simultaneous treatment in combination with different doses of TQ for 24 h.</td>
<td>83</td>
</tr>
<tr>
<td>10.</td>
<td>Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone and post treatment in combination with different doses of TQ for 24 h.</td>
<td>87</td>
</tr>
</tbody>
</table>
11. The apportionment of structural aberrations in Wistar rat bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of QCT pre-treatment.

12. The apportionment of structural aberrations in Wistar rat bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of QCT simultaneous treatment.

13. The apportionment of structural aberrations in Wistar rat bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of QCT post treatment.

14. Pre, simultaneous and post treatment of QCT - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

15. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone or in combination of pre-treatment with different doses of QCT for 24 h.

16. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone or in combination of simultaneous treatment with different doses of QCT for 24 h.

17. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone or in combination of post treatment with different doses of QCT for 24 h.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Titles</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Graphical representation of bone marrow cells containing chromosome aberrations after treatment with increasing doses of FeSO₄ for 24 h.</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>Photomicrographs illustrating the effects of iron sulfate (FeSO₄) on bone marrow cells of Wistar rats (A) Chromatid break; (B) Ring chromosome, Acentric chromosome; (C) Chromatid break; (D) Chromosome break and Tri radial formation.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Mean number of micronucleated polychromatic erythrocytes (MNPCes) in 2000 polychromatic erythrocytes (PCEs) in animals treated with increasing doses of FeSO₄ for 24 h.</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>Representative images of polychromatic erythrocytes (PCEs) containing micronuclei in bone marrow smear of Wistar rats after 24 h of iron sulfate treatment. [A, B, C, D, F, G, H, I] micronucleus in polychromatic (immature) erythrocyte, bluish in colour. [E]: micronucleus in normochromatic (matured) erythrocytes.</td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>DNA damage induced by FeSO₄ in bone marrow cells after treatment with increasing doses of FeSO₄ for 24 h assessed through comet parameter: tail moment.</td>
<td>62</td>
</tr>
<tr>
<td>5b</td>
<td>DNA damage induced by FeSO₄ in bone marrow cells after treatment with increasing doses of FeSO₄ for 24 h assessed through comet parameter: olive tail moment.</td>
<td>62</td>
</tr>
<tr>
<td>6</td>
<td>SCGE (Comet) pictures illustrating the effects of iron sulfate (FeSO₄) on the extent of DNA damage. [A] Negative control (DMSO); [B] Positive Control (CP); [C] treated with FeSO₄ at the dose of 50 mgFe/kg; [D] treated with FeSO₄ at the dose of 100 mgFe/kg; [E] treated with FeSO₄ at the dose of 200 mgFe/kg respectively.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Broken-line relationship of pre-treatment of TQ levels with FeSO₄ to reduction in Chromosomal Aberrations in bone marrow cells of Wistar rats.</td>
<td>65</td>
</tr>
</tbody>
</table>
8 Broken-Line relationship of simultaneous treatment of TQ levels with FeSO\textsubscript{4} to reduction in Chromosomal Aberrations in bone marrow cells of Wistar rats.

9 Broken-line relationship of post treatment of TQ levels with FeSO\textsubscript{4} to reduction in Chromosomal Aberrations in bone marrow cells of Wistar rats.

10 Photomicrographs illustrating the effects of iron sulfate (FeSO\textsubscript{4}) and thymoquinone (TQ) on bone marrow cells of Wistar rats (A) Chromatid break; (B) Dicentric chromosome; (C) Ring chromosome; (D) Acentric fragment.

11 Broken-line relationship of Pre- treatment with TQ levels to reduction in iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

12 Broken-line relationship of simultaneous treatment with TQ levels to reduction in iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

13 Broken-line relationship of post treatment with TQ levels to reduction in iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

14 Representative images of polychromatic erythrocytes (PCEs) containing micronuclei in bone marrow smear of Wistar rats after 24 h of iron sulfate treatment along with different doses of thymoquinone. [A, B, C, D, E, G, H, I] micronucleus in polychromatic (immature) erythrocyte, bluish in colour. [F]: micronucleus in normochromatic (matured) erythrocytes.

15a Broken-line relationship of pre-treatment of TQ levels with FeSO\textsubscript{4} to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

15b Broken-line relationship of pre-treatment of TQ levels with FeSO\textsubscript{4} to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.
16 SCGE (Comet) pictures illustrating the effects of iron sulfate and thymoquinone (TQ) pre-treatment on the extent of DNA damage. [A] Negative control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with TQ at the dose of 6, 9, 12, 15, 18, 21 and 24 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.

17a Broken-line relationship of simultaneous treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

17b Broken-line relationship of simultaneous treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

18 SCGE (Comet) pictures illustrating the effects of iron sulfate and thymoquinone (TQ) simultaneous treatment on the extent of DNA damage. [A] Negative control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with TQ at the dose of 6, 9, 12, 15, 18, 21 and 24 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.

19a Broken-line relationship of post treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

19b Broken-line relationship of post treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

20 SCGE (Comet) pictures illustrating the effects of iron sulfate and thymoquinone (TQ) post treatment on the extent of DNA damage. [A] Negative control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with TQ at the dose of 6, 9, 12, 15, 18, 21 and 24 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.

21 Broken-line relationship of pre-treatment of QCT levels with FeSO₄ to reduction in Chromosomal Aberrations in bone marrow cells of Wistar rats.
22 Broken-line relationship of simultaneous treatment of QCT levels with FeSO$_4$ to reduction in Chromosomal Aberrations in bone marrow cells of Wistar rats.

23 Broken-line relationship of post treatment of QCT levels with FeSO$_4$ to reduction in Chromosomal Aberrations in bone marrow cells of Wistar rats.

24 Photomicrographs illustrating the effects of iron sulfate (FeSO$_4$) and quercetin (QCT) on bone marrow cells of Wistar rats (A) Acentric fragment; (B) Chromosome break; Ring chromosome (C) Acentric fragment (D) Dicentric chromosomes.

25 Broken-line relationship of pre-treatment of QCT - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

26 Broken-line relationship of simultaneous treatment of QCT - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

27 Broken-line relationship of post treatment of QCT - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.

28 Representative images of polychromatic erythrocytes (PCEs) containing micronuclei in bone marrow smear of Wistar rats after 24 h of iron sulfate treatment along with different doses of quercetin.

29a Broken-line relationship of pre-treatment of QCT levels with FeSO$_4$ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

29b Broken-line relationship of pre-treatment of QCT levels with FeSO$_4$ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.
SCGE (Comet) pictures illustrating the effects of iron sulfate and quercetin (QCT) pre-treatment on the extent of DNA damage. [A] Negative control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.

Broken-line relationship of simultaneous treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

Broken-line relationship of simultaneous treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

SCGE (Comet) pictures illustrating the effects of iron sulfate and quercetin (QCT) simultaneous treatment on the extent of DNA damage. [A] Negative control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.

Broken-Line relationship of post treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

Broken-Line relationship of post treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

SCGE (Comet) pictures illustrating the effects of iron sulfate and quercetin (QCT) post treatment on the extent of DNA damage. [A] Negative control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
Chapter 1

Introduction

Iron
ABSTRACT

Iron poisoning is an iron overload caused by an excess of iron intake and usually refers to an acute overload, which promotes deleterious effect at cellular and tissue levels, leading to the cell death, tissue necrosis and degenerative diseases or cell phenotype changes and cancer formation. Iron catalyzes generation of hydroxyl radicals which intensify oxidative stress. To ameliorate such damaging effects several natural and herbal products including thymoquinone (TQ) and quercetin (QCT), have been evaluated in the recent past. TQ is a promising bioactive phytochemical compound which is found in the seeds of *Nigella sativa* plant. It has recently attached significant scientific attention due to its potent *in vitro* and *in vivo* anticancer and effective antioxidant properties. QCT, one of the major flavonoids in some fruits and vegetables, has much stronger antioxidative and anticarcinogenic activities. To further evaluate the ameliorative property of TQ and QCT against genotoxicity induced by iron, TQ was injected with increasing doses (6, 9, 12, 15, 18, 21 and 24 mg/kg) and QCT was injected with increasing doses (125, 250, 375, 500, 625, 750 and 875 mg/kg) for pre, simultaneous and post treatment. Bone marrow chromosomal aberrations assay, micronucleus assay and alkaline version of the comet assay were done to assess the DNA damage, served as endpoints of genotoxicity. The first part was done to determine the maximum toxic dose of iron sulfate among the doses of 50, 100 and 200 mgFe/kg and the second part was done to observe the optimum level of TQ and QCT on chromosome aberrations, micronucleus and comet assay induced by FeSO₄, 200 mg Fe/kg. The study revealed significant anti genotoxic effects of TQ and QCT and the best response was observed at the dose of 18 mg/kg of TQ and 500 mg/kg dose of QCT in decreasing iron induced chromosomal aberrations, yields of micronuclei and DNA damage while at the doses of 21 and 24 mg/kg of TQ and 625, 750 and 875 mg/kg doses of QCT, the less protective effect may be attributed to its pro-oxidant properties at higher doses. TQ and QCT showed the most efficient anticlastogenic effect during simultaneous treatment with iron sulfate. The study confirms the antioxidant and ameliorative properties of TQ and QCT.
INTRODUCTION

1. Introduction

Humans are daily exposed to numerous chemicals, like those present in food additives, packing materials, drugs, cosmetics and pesticides. It is of vital importance that chemicals present in those products are evaluated for their potential adverse health effects for humans before marketing. Many of these chemicals are genotoxic and can cause DNA damage, which can form a major threat to the integrity of chromosomes and viability of cells. Fortunately, cells are equipped with several DNA repair mechanisms, which can repair/remove the different types of DNA lesions efficiently, and accurately maintain the integrity of the genome (Friedberg et al., 1995; Van Steeg, 2001; Camenisch and Naegeli, 2009). Defects in DNA repair give rise to an increase in sensitivity to DNA-damaging agents, accumulation of mutations, various metabolic disorders, apoptosis, cell death, accelerated ageing, genetic diseases or development of cancer (Christmann et al., 2003; Kirkland et al., 2005; Camenisch and Naegeli, 2009). Through the years several well-defined tests have been developed for the assessment of harmful effects of chemicals and drugs. 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). Iron poisoning is an iron overload caused by an excess of iron intake and usually refers to an acute overload which promotes deleterious effect at cellular and tissue levels, leading to the cell death, tissue necrosis and degenerative diseases or cell phenotype changes and cancer formation. To ameliorate such damaging effects several natural and herbal products including thymoquinone (TQ) and quercetin (QCT), have been evaluated in the recent past. TQ is a promising bioactive phytochemical compound which is found in the seeds of *Nigella sativa* plant. It has recently attached significant scientific attention due to its potent *in vitro* and *in vivo* anticancer and effective antioxidant properties. QCT, one of the major flavonoids in some fruits and vegetables, has much stronger antioxidative and anticarcinogenic activities. To further evaluate the ameliorative property of TQ and QCT against genotoxicity induced by iron, First the focus in this thesis lies in determining the genotoxicity of iron sulfate and second was done to observe the ameliorative property.
and to obtain the optimum level of TQ and QCT on genotoxicity induced by iron sulfate. Over the past decades genetic toxicology testing has demonstrated that no single test is capable to detect both types of genotoxic effects, chromosome aberrations and gene mutations. Therefore, the potential genotoxic effects of chemicals are assessed in a battery of in vitro and in vivo tests. Genotoxic tests are usually performed in the early development of a chemical, since these are comparatively short in duration, relatively inexpensive and help to identify potential genotoxic carcinogens, which otherwise would only be known after completion of the 2-year cancer bioassay (Witte et al., 2007). There are several strategies for genotoxicity testing of chemicals and drugs. The guidance for drugs and chemicals is different from each other. In 1981 in Europe a law was introduced which made it compulsory to test both new and existing chemicals for their potential harmful effects for humans. Little information is available on toxicity, however, for 99 percent of the enormous number of existing chemicals placed on the market before 1981. A large number of these chemicals are still being used and at very low levels they are not considered to be harmful to human health. However, if these substances accumulate in the human body, dangerous concentrations can be reached and consequently may have adverse effects on human health. It is important to pay attention to the effects of genotoxic compounds, since DNA damage induced by these agents may lead to cancer and other genetic diseases. Genotoxicity has two different endpoints: gene mutations such as base pair substitution, frame shifts, deletions and insertions, and chromosome aberrations, which in turn can be structural (clastogenic effect) and/or numerical (aneugenic effect). Through the years, several in vitro and in vivo tests have been developed for genotoxic screening of chemicals. The most commonly used in vivo conditions are several transgenic animal models to detect chemicals causing gene mutations, and the in vivo micronucleus test and the in vivo chromosomal aberration tests to detect chemicals causing chromosomal aberrations. It is now widely accepted that no single test selected from the wide range available can be expected to fulfill the requirements of simplicity, rapidity and low cost and yet be absolutely accurate in predicting genotoxic effects to humans. However, there is considerable and growing evidence that a judicious combination of test procedures affecting different genetic endpoints will detect the majority of potential mutagens (Shelby, 1988).
A set of in vitro and in vivo genotoxicity tests with different endpoints have been established for assessment of genotoxic potential of chemicals as shown in Table 1. In this part of thesis, we restrict our discussion to the tests considered valid and necessary by the ICH (International Conference of Harmonization Tripartite Guidelines) process (1997), which in turn is accepted by all government agencies worldwide.

Table 1. Tests and End-Points

<table>
<thead>
<tr>
<th>Mutagenic Process End</th>
<th>Points Testing</th>
<th>Mutagenic Process End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premutagenic lesions</td>
<td>Interaction of chemical and DNA</td>
<td>DNA adducts</td>
</tr>
<tr>
<td>DNA damage</td>
<td>DNA damage and repair</td>
<td>Comet assay</td>
</tr>
<tr>
<td>Fixed in gene mutation</td>
<td>Gene mutation including base pair substitutions and frameshifts</td>
<td>Ames test E. coli substitutions and frameshifts WP2 tryptophan reversion assay in vivo genetic assays</td>
</tr>
<tr>
<td>Alteration of DNA</td>
<td>Chromosome aberrations, DNA breakage</td>
<td>Chromosome aberration assay Micronucleus assay</td>
</tr>
</tbody>
</table>

1.1. Genomic instability

Genomic instability refers to an increased tendency of alterations in the genome during the life cycle of cells. It is a major driving force for tumorigenesis. “Genomic instability is defined as a process prone to genomic changes or an increased propensity for genomic alterations. During cell division, genomic instability is associated with the failure of parental cells to accurately duplicate the genome and precisely distribute the genomic material among the daughter cells.” The ultimate goal of cell division for most non-cancerous somatic cells is to accurately duplicate the genome and then evenly divide the duplicated genome into the two daughter cells. This ensures that the daughter cells will have exactly the same genetic material as their parent cell. Failure to achieve this purpose, or abnormally high-frequency of errors during this process will result in various
forms of genome alterations in the daughter cells. Those alterations include, but are not limited to, various forms of mutations on specific genes, amplifications, deletions or rearrangements of chromosome segments, gain or loss of an entire chromosome(s), etc. Accumulation of these genomic alterations may cause dysregulation of cell division, imbalance between cell growth and death, and cancer. In the present work structural genomic instabilities have been taken into consideration.

1.2. Iron

Iron is an absolute requirement for most forms of life, including humans and most bacterial species, because plants and animals all use iron. Iron is essential to life because of its unusual flexibility to serve as both an electron donor and acceptor. Iron can also be potentially toxic. Its ability to donate and accept electrons means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide into free radicals. Free radicals can cause damage to cellular membranes, proteins, and DNA, a wide variety of cellular structures, and ultimately kill the cell. To prevent that kind of damage, all life forms that use iron bind the iron atoms to proteins. That allows the cells to use the benefits of iron, but also limit its ability to do harm (Andrews, 1995).

1.2.1. Iron – the element

Ferrous sulfate is blue green crystals, which is soluble in water. Iron can alter between two different oxidation states, Fe$^{2+}$ (ferrous iron) and Fe$^{3+}$ (ferric iron). This ability provides iron with a precious quality in biochemistry, namely the ability to accept or donate an electron (Figure 1). This ability is the reason for the role of iron in, not only oxygen transport by hemoglobin which is the main function of iron in the body and the most widely known, but also DNA synthesis and energy production. However, the chemical properties of iron are also an obstacle when it comes to the availability. Due to the drive to decrease free energy, iron easily oxidizes to ferric (trivalent, Fe$^{3+}$) iron, which in turn precipitates as the insoluble iron hydroxide at pH 7. Thus, in spite of the essential biological importance the major part of the iron in our environment is insoluble, making it unavailable for biological purposes. As a consequence, the human body has since the beginning of time, in interplay with our environment, adapted to this by developing a very limited capacity of secrete/lose iron. The only significant physiological loss of iron, apart from menstruation losses, is the one taking place when worn out enterocytes is being sloughed.
1.2.2. Iron—the nutrient

When going from being "just" an element to being a nutrient, iron passes over to be characterized as either heme iron or non-heme iron. The iron in heme iron is incorporated into a protoporphyrin skeleton forming the most beautiful heme molecule, which in turn is a functional part of, i.e. cytochromes, myoglobin and hemoglobin. The "father" of biochemistry, Felix Hoppe-Seyler (1825-1895), was the first to characterize the crystallized structure of hemoglobin, and its ability to bind oxygen. The basis for the diet based characterization as heme iron or non-heme iron lies in the stability of the structure the iron is incorporated in. Due to its ability to donate electrons iron is also able to catalyze Fenton and Haber-Weiss reactions, which create reactive oxygen species. To protect various structures in the organism from oxidative stress, the nonfunctional part of the iron pool is bound to different molecules, such as transport and storage proteins (e.g. transferrin and ferritin). The iron of the functional iron pool is part of numerous enzymes (e.g. catalases and ribonucleotide reductase) and heme proteins. When these iron containing structures, functional and nonfunctional, are present in the diet they are degraded by our digestive system. However, the heme structure strongly resists degradation. Thus, when presented to the mucosa cell layer in the intestines the iron in the heme is taken up as a complex molecule whereas the non-heme iron is taken up in its elemental form. As a consequence of lacking a protective "shell" like protoporphyrin, the absorption of non-heme iron is substantially affected by the composition of the diet and the iron status, whereas the heme iron is much less affected by these factors (Hallberg, et al., 2000b, Hunt 2005). The inorganic non-heme iron is the most dominant form of iron in our diet. It is predominantly found in vegetable foods, mainly cereals, but also present in animal products. Although meat is known as a provider of heme-iron, the major part of iron is in the form of non-heme. In general, the proportions of heme iron in red meat range from ~25-50%. However, when incorporating chicken or fish into a meal or diet the contribution of heme iron will be negligible (Hallberg, et al., 2000).
1.2.3. Iron Chemistry

Iron is of fundamental importance for the growth, development and well being of almost all living organisms. Multiple biological systems have been developed for the uptake, utilization, storage and homeostasis of iron in microbes, plants and mammals. Its bioavailability is generally limited and higher species often exhibit deficiency states. Paradoxically, iron overload conditions occur. In quantitative terms, iron is the most important essential trace element, being an important mineral for essential nutrition. Adult men and women contain approximately 55 and 45mg per kilogram of body weight of iron, respectively (Halliwell, 1989). Iron is a d-block transition element that can exist in oxidation states ranging from -2 to +6. Within biological systems, iron exists in 3 oxidation states, namely ferrous (+2), ferric (+3) and ferryl (+4) states. Iron participates in reactions where there is a transfer of electrons (redox reactions), and in so doing it can reversibly bind to ligands by virtue of its unoccupied d orbitals. The electronic spin state and biological redox potential of iron can change according to the ligand to which it is bound. Iron is therefore particularly suited to participate in a large number of biochemical reactions (Webb, 1992). When iron is complexed with water, it is readily hydrolysed and polymerized at a physiological pH of 7 (Spiro and Saltman, 1974). When water molecules are replaced by other chelating ligands, stable complexes are formed. Under conditions of neutral or alkaline pH, iron is found in the Fe$^{3+}$ state and at an acidic pH the Fe$^{2+}$ state is favoured. The strongest complexes of Fe$^{3+}$ tend to be with oxygen donor ligands, e.g. citrates, phosphates, phenols or carbohydrates, whereas Fe$^{2+}$ prefers nitrogen or nitrogen with oxygen donors. The complexes formed with Fe$^{3+}$ are large, have poor solubility and upon their aggregation lead to pathological consequences (Halliwell, 1989).

1.2.4. Body Iron and Its Physiological Role

Most well-nourished people have 3 to 5 grams of iron in their bodies. Of this, about 2.5 g is contained in the hemoglobin needed to carry oxygen through the blood, and most of the rest (approximately 2 grams in adult men, and somewhat less in women of child bearing age) is contained in ferritin complexes that are present in all cells, but most common in bone marrow, liver, and spleen. The liver's stores of ferritin are the primary physiologic source of reserve iron in the body. The reserves of iron in adults tend to be lower in children and women of child-bearing age, than in men and in the elderly.
Women who must use their stores to compensate for iron lost through menstruation, pregnancy or lactation, have lower body stores, which may consist of 500 mg or even less (Schrier and Bacon, 2005).

Of the body's total iron content, about 400 mg is devoted to cellular proteins that use iron for important cellular processes like storing oxygen (myoglobin), or performing energy-producing redox reactions (cytochromes). A relatively small amount (3-4 mg) circulates through the plasma, bound to transferrin (Fleming and Bacon, 2005). Because of its toxicity, free soluble iron (soluble ferrous ions Fe(II)) is kept in low concentration in the body. The most important group of iron-binding proteins contains the heme molecules, all of which contain iron at their centers. Humans and most bacteria use variants of heme to carry out redox reactions and electron transport processes. These reactions and processes are required for oxidative phosphorylation. That process is the principal source of energy for human cells; without it, most types of cells would die. The iron-sulfur proteins are another important group of iron-containing proteins. Some of these proteins are also essential parts of oxidative phosphorylation. Humans also use iron in the hemoglobin of red blood cells, in order to transport oxygen from the lungs to the tissues and to export carbon dioxide back to the lungs. Iron is also an essential component of myoglobin to store and diffuse oxygen in muscle cells (Fleming and Bacon, 2005).

The human body needs iron for oxygen transport. That oxygen is required for the production and survival of all cells in our bodies. Human bodies tightly regulate iron absorption and recycling. Iron is such an essential element of human life, in fact, that humans have no physiologic regulatory mechanism for excreting iron. Most humans prevent iron overload solely by regulating iron absorption. Those who cannot regulate absorption well enough get disorders of iron overload. In these diseases, the toxicity of iron starts overwhelming the body's ability to bind and store it (Schrier and Bacon, 2005).

**1.2.5. Body iron homeostasis**

**1.2.6. Iron Absorption and Distribution in Humans**

The human body contains approximately 3–5 g of iron (45–55 mg/kg of body weight in adult women and men, respectively), distributed as illustrated in Figure 2. The majority of body iron (~60–70%) is utilized within hemoglobin in circulating red blood cells (Ponka, 1997; Andrews, 1999). Other iron-rich organs are the liver and muscles. Approximately 20–30% of body iron is stored in hepatocytes and in reticuloendothelial
macrophages, to a large extent within ferritin and its degradation product hemosiderin. The remaining body iron is primarily localized in myoglobin, cytochromes, and iron containing enzymes. A healthy individual absorbs daily 1–2 mg of iron from the diet, which compensates nonspecific iron losses by cell desquamation in the skin and the intestine. In addition, menstruating women physiologically lose iron from the blood. Erythropoiesis requires approximately 30 mg iron/day, which is mainly provided by the recycling of iron via reticuloendothelial macrophages. These ingest senescent red blood cells and release iron to circulating transferrin. The pool of transferrin-bound iron (~3 mg) is very dynamic and undergoes >10 times daily recycling. Mammals do not possess any physiological pathway for iron excretion. Thus, body iron homeostasis is regulated at the level of iron absorption. Misregulated iron absorption leads to iron deficiency or overload.

![Diagram of iron distribution in the adult human body.](image)

**Figure 2. Iron Distribution in the Adult Human Body.**

1.3. Toxicity of Iron

1.3.1. Iron Induced Free Radicals Which Leads to Oxidative Stress

The ability of transition metal ions to undergo facile 1-electron oxidation or reduction makes them obvious potential chemical partners for reactions involving biological free radicals. It is not coincidental that superoxide dismutases, the enzymes that catalytically destroy the superoxide radical (O$_2$)$^-$ by alternately oxidizing and reducing it, have been
found containing 3 different transition metals (Cu, Mn, or Fe) at their active sites. Iron is by far the most abundant transition metal in the human body because of its roles in oxygen binding and transport and electron transport. Because of the central and essential roles of iron in the metabolisms of all aerobic organisms, humans have evolved some peculiar ways of dealing with it. These peculiarities provide opportunities for the cause of diseases related to iron absorption, transport, and metabolism, as well as for the exacerbation of general mechanisms of disease involving free radical injury. The efficiency of Fe(II) as an electron donor and of Fe(III) as an electron acceptor, with a redox potential compatible with the constraints of the cellular environment, is a fundamental feature for many biochemical reactions and renders iron to an essential mineral and nutrient. However, this very property turns iron into a potential biohazard, because under aerobic conditions, iron can readily catalyze the generation of noxious radicals. Iron’s toxicity is largely based on Fenton and Haber-Weiss chemistry (Figure 3A), where catalytic amounts of iron are sufficient to yield hydroxyl radicals (OH) from superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), collectively known as “reactive oxygen intermediates” (ROIs) (Halliwell and Gutteridge, 1990). Importantly, ROIs are inevitable byproducts of aerobic respiration and emerge by incomplete reduction of dioxygen in mitochondria. ROIs can also be generated during enzymatic reactions in other subcellular compartments, such as in peroxisomes, the endoplasmic reticulum, or the cytoplasm. ROIs are also produced by the membrane-bound NADPH oxidase complex (Hampton et al., 1998), a multisubunit enzyme primarily expressed in phagocytic neutrophils and macrophages, but also in other cell types. NADPH oxidase is an important tool for the antimicrobial defense of the organism. The enzyme complex assembles upon infection and generates high levels of superoxide in a “respiratory burst”, which is enzymatically and spontaneously dismutated to hydrogen peroxide. The reaction products give rise to more potent oxidants such as peroxynitrite (ONOO') and hypochlorite (OCI), which amplify the bactericidal and cytotoxic capacity of phagocytic cells and constitute major toxic species in vivo (Ischiropoulos and Beckman, 2003). The former is generated by the spontaneous reaction of superoxide with NO, while the latter is synthesized from hydrogen peroxide and chloride in a reaction catalyzed by myeloperoxidase. In this milieu, redox active iron catalyzes the generation of not only hydroxyl radicals, but also of organic reactive species, such as peroxyl (ROO'), alkoxyl (RO'), thyl (RS), or thylperoxyl (RSOO') radicals (Figure 3B).
Figure 3. (A) Iron-catalyzed generation of the hydroxyl radical via the Fenton reaction; the net Haber-Weiss reaction is also indicated. (B) Iron-catalyzed generation of organic radicals. (C) Heme-catalyzed generation of oxygen radicals via oxoferryl intermediates. (D) Direct interaction of iron with oxygen (Papanikolaou and Pantopoulos, 2005).

Interestingly, heme iron (either “free” or within hemoproteins) may also catalyze the formation of radicals, mainly via formation of oxoferryl intermediates (Ryter and Tyrrell, 2000) (Figure 3C). Finally, ferrous iron can also contribute as a reactant, rather than as a catalyst, to free radical generation by a direct interaction with oxygen, via ferryl (Fe$^{2+}$-O) or perferryl (Fe$^{2+}$-O$_2$) iron intermediates (Figure 3D). It has been proposed that when [O$_2$]/[H$_2$O$_2$] >100, these reactions may represent an important source for free radical generation in vivo (Huang, 2003). Free radicals are highly reactive species and may promote oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. Likewise, reactive nitrogen species, such as peroxynitrite, may lead to
protein damage via nitration. An increase in the steady state levels of reactive oxygen (and nitrogen) species beyond the antioxidant capacity of the organism, called oxidative (and nitrosative) stress, is encountered in many pathological conditions, such as chronic inflammation, ischemia–reperfusion injury, or neurodegeneration (Ischiropoulos and Beckman, 2003). Excess of redox active iron (as well as copper) aggravates oxidative (and nitrosative) stress and leads to accelerated tissue degeneration (Figure 4).

![Figure 4. Free radicals and their relationship to body injuries.](image)

1.3.2. Iron must be handled very carefully due to its ability to catalyze potentially destructive redox chemistry

In healthy cells, iron ions are never found in a naked state, but are always tightly chelated, usually by proteins. If iron is being transported or stored it must be chelated in very specific ways that discourage redox cycling (e.g., by transferrin or ferritin). When iron is allowed to redox cycle (e.g., as in cytochromes or peroxidases), it is tightly held in the context of the protein’s active site. Free iron is loose cannon, chemically (McCord, 1998). One of the most devastating actions of free redox active iron within the cell is the initiation of lipid peroxidation. Lipid peroxidation is a free radical chain reaction between polyunsaturated fatty acyl groups in cell membranes and molecular oxygen. It leaves in its wake dysfunctional membranes and cell death. Perhaps the most interesting
iron containing proteins are those that may release free redoxactive iron when a free radical-mediated 1-electron reduction occurs (ferritin, aconitase) or that may take on new biologic activity, such as the transformation of cytosolic aconitase into iron-responsive protein-1, a protein that can modulate the efficiency of translation of mRNA encoding the iron-storage protein ferritin. Thus, on a small scale the free radical-induced liberation of iron from an iron-binding protein may reflect an evolved signaling pathway; on a larger scale, however, it may result in the wholesale destruction of the organism.

1.3.3. Genotoxicity of Iron

Several studies have been conducted to demonstrate the potential induction of DNA aberrations by iron (Fe) and also by drugs and compounds containing this metal. However, the results are inconclusive, and its toxicity and mutagenic effect is still incompletely understood. Organic Fe may increase the genotoxic effects of other compounds when they are combined (WHO, 1998). For example, the mutagenic activity by doxorubicin is significantly increased within this metal, as evaluated by the Ames test (Kostoryz and Yourtee, 2001). Furthermore, Jurkat cells simultaneously treated with hydrogen peroxide and desferrioxamine (Fe chelator) significantly inhibit DNA damage, indicating that intracellular Fe, which is a redoxactive metal, plays a role in the induction of DNA strand breaks induced by hydrogen peroxide (Barbouti et al., 2001).

High levels of chromosome and chromatid aberrations were found in human lymphocytes and TK6 lymphoblast cells exposed to high-energy iron ions (56Fe) (Evans et al., 2001; Evans et al., 2003). Significant DNA damage was detected, by microgel electrophoresis, in differentiated human colon tumor cells (HT29 clone 19A) treated with ferric-nitrioltriacetate (Fe-NTA) (Glei et al., 2002). Mutagenic activity was also found in elemental and salt forms of Fe, evaluated by mutagenicity tests in Salmonella typhimurium and L5178Y mouse lymphoma cells (Dunkel et al., 1999).

Iron compounds have also been reported to be mutagenic in mammalian cells, as detected by the Syrian hamster embryo cell transformation/viral enhancement assay (Heidelberger et al., 1983), sister chromatid exchange (SCE) in hamster cells (Tucker et al., 1993) and base tautomerization in rat hepatocyte cultures (Abalea et al., 1999).

Few or no DNA damage (detected by the comet assay) occurred after treatment of human lymphocytes with ferric chloride (FeCl3) and ferrous chloride (FeCl2), all of them known
to be iron compounds (Anderson et al., 2000a, 2000b). Also, low concentrations of either Fe$^{2+}$ or Fe$^{3+}$ were not mutagenic in Chinese hamster ovary cells (CHO-9) treated in vitro, and the mitotic index was also unaffected when compared to negative control. In the other hand, high concentrations of ferrous sulfate, induces significant DNA damage, probably as a consequence of chemical contamination of the metal salt (Antunes et al., 2005).

Mutagenic potential of metallic agents used in dietary supplementation, including iron sulfate, was also investigated by means of the comet assay. Franke et al. (2006), reported a genotoxic effect of this metal in mouse blood cells after 24 h of treatment, at different concentrations. Genotoxic effects of Fe were also reported by Garry et al. (2003) in rats treated with iron oxide (Fe$_2$O$_3$) for 24 h. They observed that this metal only showed mutagenic potential when the animals were simultaneously treated with benzopyrene.

Furthermore, Hasan et al., (2005) reported that ferritin, an ubiquitously distributed iron storage protein, interacts with microtubules in vitro. In a study conducted by Maenosono et al. (2007) the bacterial reverse mutation assay using S. typhimurium was weakly positive for water-soluble FePt nanoparticles capped with tetramethyl ammonium hydroxide. Mice subchronically exposed to 33.2 mg/Kg Fe displayed genotoxic effects in whole blood in the alkaline version of the comet assay, with a significant increase in the hepatic level of Fe (Prá et al., 2008).

High-energy iron ions (LET=151 keV/microM) could induce chromosomal aberrations (measured using the fluorescence whole-chromosome painting technique) in normal and repair-deficient human fibroblasts cell lines (George et al., 2009).

Park and Park (2011) screened the potential toxicity of various iron-overloads on human leukocytes using comet assay. Ferric-nitrilotriacetate (Fe-NTA), FeSO$_4$, hemoglobin and myoglobin were not cytotoxic in the range of 10-1000 microM by trypan blue exclusion assay. The exposure of leukocytes to Fe-NTA (500 and 1000 microM), FeSO$_4$ (250-1000 microM), hemoglobin (10 microM) and myoglobin (250 microM) for 30 min induced significant DNA damage. Iron-overloads generated DNA strand break were rejoined from the first 1h, but no genotoxic effect was observed at 24h. Recently, a research group conducted an in vitro study aiming to investigate the genotoxic, clastogenic and cytotoxic effects of FeSO$_4$ in different phases of the cell cycle, using short-term cultures.
of human lymphocytes. The bioactivity parameters tested were the mitotic index, chromosomal aberrations and DNA damage index as detected by the comet assay. Our results showed that Fe induces alterations and inhibition of DNA synthesis, which together explains the concomitant occurrence of mutagenicity and cytotoxicity (Lima et al., 2008).

1.3.4. The Roles of Iron in Health and Diseases

In the healthy state, there should never be an over accumulation of free iron. Unfortunately, although humans have an intricate mechanism for controlling intestinal absorption of iron, they lack a mechanism (other than bleeding) for elimination of grossly excessive quantities. The manifold ways in which acquired iron exceeds physiologically appropriate needs are summarized in section 1.4. For the past sixty years, some merchandisers of processed foods have claimed that “iron fortified” foods will make us healthier and stronger. Unhappily, this is true only for the small minority of persons who truly are iron deficient. In developed countries, accumulation of excess iron in males can begin in early adulthood and then increase almost linearly with age. Females delay over accumulation by menstruation and/or pregnancy. Postmenopausal women can attain parity with men in iron burden within a few decades. As humans acquire the perilous metal, they are forced to contain it (in ferritin/ hemosiderin) within cells in a great variety of tissues. These include, but are not limited to, brain, heart, liver, pancreas, pituitary, joints, bone, lung, spleen and skin.

1.4. Some conditions that compromise the iron withholding defense system (Weinberg E.D. and Miklossy J, 2008).

1.4.1. Genetic disorders

Aceruloplasminemia, African siderosis, hemochromatosis, transfusion dependent: myelodysplasia, sickleemia, thalassemia.

1.4.2. Behavioral factors

- Ingestion of excessive amounts of: heme (red meat), iron supplements, ascorbic acid,
- ethanol, food that has been adulterated with iron
- Inhalation of iron-containing items: asbestos, coal, ferriferous ores & metals, tobacco
- smoke urban & subway air particulates
- Injection of excessive amounts: iron saccharate, whole blood, erythrocytes
1.4.3. Pathological conditions

Release of body iron into plasma: efflux of erythrocyte iron in hemolytic conditions, efflux of hepatocyte iron in hepatitis, loss of spleen myelo-ablative conditioning prior to cell/tissue transplant.

Iron now is recognized to be a serious risk factor for endocrinological, gastrointestinal, infectious, neoplastic, neurodegenerative, obstetric, ophthalmic, orthopedic, pulmonary and vascular diseases. Moreover, the metal accelerates development of sarcopenia and, as well, can be teratogenic (section 1.5). Increasingly, it is becoming apparent that “life was designed to exist at the very interface of iron deficiency and iron sufficiency. (Connor and Ohio, 2009)” Iron toxicity ensues from two distinct attributes of the metal. The first is its ability, in redox active form, to generate oxygen-based free radicals. Although short-lived, the latter can destroy proximate cells by initiating lipid peroxidation, enzyme denaturation, polysaccharide depolymerization and DNA strand rupture (Galaris et al., 2008). The second attribute of iron that potentiates disease is its role as an essential growth factor for nearly all pathogenic bacteria, fungi and protozoa (Weinberg, 2009) and for all neoplastic cells (Weinberg, 1996). In response, hosts have evolved a highly structured iron withholding defense system that continuously attempts to purge ‘free’ iron from all body tissues. An imbalance of these systems increases susceptibility to oxidative damage, resulting in mutations, cancer, neurological diseases, iron overload and iron deficiency related diseases.
1.5. Diseases for which excessive/misplaced iron can be a risk factor (Weinberg E.D. and Miklossy J, 2008).

**Cardiovascular**
- atherosclerosis
- cardiomyopathy
- hypertension
- ischemic stroke
- venous leg ulcer

**Dermatologic**
- porphyria cutanea tarda

**Endocrine**
- diabetes
- endometriosis
- growth deficiency
- hypogonadism
- hypothyroidism

**Hepatic**
- cirrhosis
- steatohepatitis
- viral hepatitis

**Infectious**
- bacterial
- fungal & protozoan

**Neurologic and neurodegenerative**
- Alzheimer's disease
- Huntington's disease
- multiple sclerosis
- Parkinson's disease
- pantothenate kinase
- prion disease
- amyotrophic lateral sclerosis
- depression
- Friedreich's ataxia
- cerebrovascular disease

**Obstetric**
- neonatal hemochromatosis
- pre-eclampsia

**Oncologic**
- breast cancer
- colorectal cancer
- hepatic carcinoma
- Kaposi sarcoma
- leukemia
- lung cancer

**Ophthalmic**
- macular degeneration

**Orthopedic**
- gout
- hemophilic synovitis
- osteoarthritis
- osteoporosis

**Otologic**
- aminoglycoside toxicity

**Pediatric**
- Down syndrome
- epilepsy
- sudden infant death syndrome

**Pulmonary**
- cystic fibrosis
- ozone lung injury
- pneumoconiosis

**Renal**
- aminoglycoside & vancomycin tox
EXAMPLES OF ACTION OF IRON IN SPECIFIC DISEASES

(Weinberg E.D. and Miklossy J, 2008)

1.5.1. Iron, by itself can initiate the disease

Cardiomyopathy, growth deficiency, hypogonadism, hypothyroidism, hemophilic synovitis, lung cancer, osteoporosis, pneumoconiosis.

1.5.2. Iron can be a cofactor in promoting the disease


1.5.3. Iron deposits are observed in disease-associated tissue sites

Basal ganglia in pantothenate kinase neurodegeneration, brain in prion disease, hepatocytes in cirrhosis, hepatocytes in steato- and viral- hepatitis, macula in macular degeneration, microglia in Huntington’s disease, mitochondria in Friedreich’s ataxia, pulmonary secretions in cystic fibrosis, soft tissue in Kaposi’s sarcoma, substantia nigra in Parkinson’s disease.

1.5.4. Body iron loading is associated with above-normal incidence of disease

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.

1.5.5. Maternal antibodies can impair fetal iron metabolism

Fetal or neonatal death in neonatal hemochromatosis.

1.5.6. Iron Induced Carcinogenesis in Humans and Animals

Iron-induced malignant tumors were first reported in 1959 by repeated intramuscular injection of iron dextran complex in rats (Richmond, 1959). Years later, sarcomas were shown in patients in whom iron preparations had been injected (Greenberg, 1976). Beginning in the 1980s, some epidemiological reports have associated increased iron
exposure with elevated cancer risk in either prospective or retro-prospective studies, by comparing cancer cases with their matched controls (Kazantzis, 1981; Graf and Eaton, 1985; Stevens et al., 1986; Selby and Friedman, 1988; Stevens et al., 1988). Iron exposure variables in those epidemiological studies included dietary iron intake, iron vitamin supplementation, body iron stores as measured by ferritin, serum iron (also known as transferrin iron), total iron binding capacity of transferrin or transferrin saturation, and gene status for hereditary hemochromatosis, an iron overload disease. Because iron stored in the iron proteins is tightly bound, and thus not readily bioavailable for adverse effects, those iron markers are not direct measures of iron that is responsible for iron toxicity. Perhaps for these reasons, epidemiological studies on the association of iron with cancer remain inconclusive (Selby and Friedman, 1988; Stevens et al., 1988; Tzeng et al., 1991; Wurzelmann et al., 1996; Bird et al., 1996). Nevertheless, the majority of existing epidemiological data supports the role of iron in human cancer and along with animal and cellular evidence suggesting that iron may be carcinogenic (Shigeru Okada, 1998).

1.6. Effect of Natural antioxidant on Free Radicals Production and Removal

There has been growing evidence over the past three decades showing that malnutrition (e.g., dietary deficiencies of protein, selenium, and zinc) or excess of certain nutrients (e.g., iron and vitamin C) gives rise to the oxidation of biomolecules and cell injury. Many naturally occurring compounds have been reported to have cancer-preventive effects (Yamagishi et al., 2001; De Stefani et al., 2004; Vuorelaa et al., 2004). Plant-derived foods, such as fruits, vegetable, herbs and spices and the their isolated phytochemicals have been claimed to have antimitagenic and anticarcinogenic activities (Nandi et al., 1997; Sarkar et al., 1997; El-Hamss et al., 1999; Guyonnet et al., 2001; Steinkellner et al., 2001; Yamagishi et al., 2001; Cavin et al., 2002; El Hamss et al., 2003; Halder et al., 2005). A systematic screening of plant extracts, food supplements or dietary products for their potential chemopreventive and antimitagenic activity against chemical carcinogens is urgently needed. A large body of the literature supports the notion that dietary antioxidants are useful radio protectors and play an important role in preventing many human diseases (e.g., cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration, and diabetes). The knowledge of enzymatic and non-enzymatic oxidative defense mechanisms will serve as a guiding principle for
establishing the most effective nutrition support to ensure the biological safety of manned space missions. To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with an extremely efficient and highly sophisticated protective system the so-called "antioxidant defensive system". It involves a variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals (Percival, 1998). A broader definition of an antioxidant is "any substance which, when present at low concentrations compared to those of oxidizable substrates, significantly delays or prevents oxidation of those substrates". The term oxidizable substrate includes DNA, lipids, proteins and carbohydrates which are the essential building blocks of a biological system (Halliwell et al., 1995). Oxidative stress occurs as a result of an increase in oxidative metabolism, which produces a number of ROS. To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects (Vaya and Aviram, 2001). High dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases such as heart diseases, cancers and cataracts.
Antioxidant Potential of Thymoquinone
1.6.1. Thymoquinone

Animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. *Nigella sativa*, commonly known as black cumin, is an annual flowering plant native to Mediterranean countries, Pakistan and India (Gali-Muhtasib *et al.*, 2006). Its seed oil had been used in Arab traditional herbal medicine for the treatment of arthritis, lung diseases and hypercholesterolemia (Khader *et al.*, 2009). Studies had shown that the biological activity of *Nigella sativa* seeds is mainly attributed to its essential oil component which is pre-dominantly (30–48%) TQ (2-isopropyl-5-methyl-1,4-benzoquinone) (Burits and Bucar, 2000; Hajhashemi,* et al.*, 2004) (Figure 5).

![Structure of TQ (2-Isopropyl-5-methyl-1, 4-benzoquinone).](image)

**Figure 5. Structure of TQ (2-Isopropyl-5-methyl-1, 4-benzoquinone).**

Since the extraction of TQ by El-Dakhakhany (1963), a number of studies have tested this compound for its therapeutic effect in many diseases including inflammation, cancer, sepsis, atherosclerosis and diabetes. These studies have revealed many different modes of action of TQ (Figure 6); however there is still insufficient data to provide conclusive evidence of its efficacy against inflammation and cancer.
1.6.1.1. Antioxidant Activity

The effect of TQ in ameliorating oxidative damage and tissue inflammation has been cited in several reports. Its broad spectrum antioxidant potential is associated with its potential to alter "redox state" and its scavenging ability against free radicals, including reactive oxygen species (ROS; superoxide anion radical, hydroxyl radical's, hydrogen peroxide, peroxynitrate) through modulation of hepatic and extra hepatic antioxidant enzymes such as superoxide dismutase, catalase, and GPx (Mansour et al., 2002; Hamdy and Taha, 2009).

Additionally, TQ reduces the cellular oxidative stress by inducing glutathione (GSH) under different experimental conditions. A substantial body of information exists, implicating a pathological state mediated through induction of lipid peroxidation. Thus far, studies cited in the literature have suggested that TQ protects the kidney against ifosfamide, mercuric chloride, cisplatin, and doxorubicin-induced damage by preventing renal GSH depletion and anti lipid peroxidation product accumulation, thereby improving renal functioning (Badary et al., 1997; Badary, 1999; Fouda et al., 2008). TQ supplementation in diet of Sprague-Dawley rats inhibited malonaldehyde content in liver, which suggests reduction in the lipid peroxide formation (Al-Johar et al., 2008) (Figure 7). The protective effects of N. sativa and TQ on cell viability and ROS
production in cultured PC-12 cells were investigated under SGD conditions by Mousavi et al. (2010). The experimental results suggest that N. sativa extract and TQ protects the PC-12 cells against SGD-induced cytotoxicity via antioxidant mechanisms mediated by inhibition of the intracellular ROS production.

Figure 7. A scheme showing free radicals and their relationship to body injuries induced by iron sulfate and the potential protective role of thymoquinone.

1.6.1.2. TQ as a free radical scavenger

Mansour et al. (2002) had reported that TQ can act as a potent free radical and superoxide radical scavenger at both nanomolar and micromolar range, respectively. This was consistent with the report by Badary et al. (2003) showing that TQ was a potent superoxide anion scavenger, and was able to inhibit iron-dependent microsomal lipid peroxidation in a dose-dependent manner. When compared to a synthetic structurally related tert-butylnhydroquinone (TBHQ), TQ was found to be more effective than TBHQ as a superoxide anion scavenger, but not as a scavenger of 2, 20-diphenylppicrylhydrazyl and hydroxyl radicals (Badary et al., 2003). These results suggest that TQ is a radical scavenger with a potential role in the prevention and treatment of oxidative stress (Figure 8).
1.6.1.3. Anti-Inflammatory and Chemopreventive Activity

Relevant to cancer and inflammation, COX-2 is an important component of the inflammatory response as well as an early response gene whose upregulation leads to the production of multiple prostaglandins (PGs). As a consequence, PGs promote tumor growth by multiple protumorigenic mechanisms such as by stimulating angiogenesis, promoting proliferation, promoting cell invasion, and inhibiting apoptosis through the activation of different oncogenes including v-src, vHa-ras, human epidermal growth factor receptor 2/neu and Wnt (Buchanan and DuBois, 2006; Wang and DuBois, 2008) (Figure 9). The first indication of the effect of TQ on in vivo production of PGs and lung inflammation in a mouse model of allergic airway inflammation was reported by El Mezayen et al. (2006). Similar to the COX-2 enzyme, another enzyme, 5-lipoxygenase (5-LOX), converts the precursor arachidonic acid molecules to hydroxyeicosatetraenoic acids or leukotrienes (LT), which in turn enhance proliferation and survival and restrains cells to undergo apoptosis. Therefore, the potential of TQ in suppressing inflammation through inhibition of LT is worth considering as a provocative area to explore in the context of cancer. Amelioration of the inflammation by TQ has been reported by El-Gouhary et al., (2005) who found a potent effect of TQ presumably occurring via induction of GSH. Studies have collectively supported the notion that TQ could be useful in intervening the inflammatory cascade, which may lead to the inhibition of cancer progression and thus improve patients' morbidity and mortality (Banerjee et. al., 2009).
1.6.1.4. Antiproliferative Activity

Of particular relevance to pathophysiology of tumor growth is proliferation of tumor cells. TQ has been shown to inhibit cell proliferation in cultured cells derived from human breast and ovarian adenocarcinoma (Shoieb et al., 2003), myeloblastic leukemia cells, HL-60 (El-Mahdy et al., 2005), squamous carcinoma [SCC VII; (Ivankovic et al., 2006)], fibrosarcoma [FSSaR; (Ivankovic et al., 2006)], laryngeal neoplastic cells-Hep-2 (Wormack et al., 2006), and prostate and pancreatic cancer (PC) cell lines (Tan et al., 2006; Kaseb et al., 2007; Yi et al., 2008; Banerjee et al., 2009). With respect to p53 mutational status, Roepke et al., (2007) evaluated the antiproliferative and proapoptotic effect of TQ in two human osteosarcoma cells lines—p53-null MG63 cells and p53-mutant MNNG/HOS cells—and concluded that despite differential involvement of the mitochondrial pathway in inducing apoptosis in these two cell lines, TQ functioned in p53-independent manner in inducing apoptosis in these human osteosarcoma cell lines. This highlights the potential importance of TQ in the clinical setting for the treatment of this cancer because loss of p53 function is frequently observed in osteosarcoma patients.

1.6.1.5. Cell Cycle Regulation

Evidence so far indicates the effectiveness of TQ in arresting tumor cells at different stages of their progression. (Shoieb et al., 2003; Gali-Muhtasib et al., 2004). Studies indicate that TQ pretreatment potentiates the arrest of cells in the progression of the cell cycle.
1.6.1.6. Apoptosis

Tumor cells tend to elude apoptosis by deregulating genes that perpetuate programmed cell death (apoptosis). Several studies to date, mostly limited to in vitro cell experiments, document TQ-mediated apoptosis by regulating multiple targets in the apoptotic machinery. Although evidence for reduced cell viability has been observed in response to TQ treatment in breast, colon, bone, leukemia, larynx, prostate, and PC cells, the classical hallmark of apoptosis such as chromatin condensation, translocation of phosphatidyl serine across plasma membrane, and DNA fragmentation have been documented in TQ-treated cells. Furthermore, TQ has been shown by others to activate the mitochondrial/intrinsic pathway that involves release of cytochrome c from the mitochondria into the cytosol, which in turn binds to the apoptosis protease activation factor-1 (ApaF-1) and leads to the activation of the initiator caspase-9. Activation of caspase-9 has been described following exposure of human myeloblastic leukemia HL-60 cells (El-Mahdy et. al., 2005) and PC cells to TQ (Banerjee et. al., 2009). Thus, one mechanism of apoptosis induction by TQ involves interference with mitochondrial integrity. In a recent published study by Torres et al. (2010) the expression of Mucin-4 (MUC-4) was investigated in pancreatic cancer cells and it was found that TQ downregulates MUC-4 expression through the proteosomal pathway and induced apoptosis by the activation of c-Jun NH2-terminal kinase and p38 mitogenactivated protein kinase (MAPK) pathways. In agreement with previous studies, the decrease in MUC4 expression correlated with an increase in apoptosis, decreased motility, and decreased migration of pancreatic cancer cells. Accordingly, MUC4 transient silencing showed that c-Jun NH2-terminal kinase and p38 MAPK pathways become activated in pancreatic cancer cells, indicating that the activation of these pathways by TQ is directly related to the MUC4 down regulation induced by the drug (Torres et al., 2010) (Figure 10).
Figure 10. Multitargeted effects of TQ. ROS, reactive oxygen species; DT, dehydrogenase quinine; COX-2, cyclooxygenase-2, PGE-2, prostaglandin E-2; Bcl-2, B-cell non-Hodgkin lymphoma-2; Bax, Bcl-2-associated x protein; CHEK-1, checkpoint kinase 1 homolog; XIAP, x-linked inhibitor of apoptosis protein; E2F-1, E2F transcription factor 1; GATA, GATA transcription factor; UHRF, ubiquitin PHD RING finger; TNF α, tumor necrosis factor alpha; IL, interleukin; MCP-1, monocyte chemotactic protein-1; Muc-4, Mucin-4; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase.

1.6.1.7. TQ Inhibits Angiogenesis

Of relevance to tumor growth, angiogenesis is a prerequisite for supplying oxygen and nutrients to sustain growth beyond a critical size and metastasis. Using the human umbilical vein endothelial cells (HUVECs) and aortic ring assay as a model of angiogenesis, it was demonstrated that TQ modulates vessel outgrowth and various steps of angiogenesis. TQ inhibits proangiogenic factor (VEGF)-induced ERK activation, and inhibited tube formation on matrigel and induced dose-dependent decrease in the proliferative activity of endothelial cells (Chehl et al., 2009). Overall, these findings indicate that TQ interferes with all essential steps of neovascularization from proangiogenic signaling to endothelial cell migration and tube formation.
1.6.1.8. *In vivo* Antitumor Activity

Despite limited studies so far, the antitumor activity of TQ seems promising both for chemoprevention as well as in preventing drug-induced toxicity. Additionally, this compound exhibits some selectivity to cancer cells, since normal cells, human pancreatic ductal epithelial cells (HPDE) and mouse keratinocytes are resistant to the apoptotic effects of TQ (Gali-Muhtasib *et al.*, 2004; Banerjee *et al.*, 2009). Below, we systemically review studies reported on the site specific antitumor effect of TQ.

1.6.1.9. Therapeutic Uses

Gali-Muhtasib *et al.* (2008) evaluated the therapeutic potential of TQ in two different murine colon cancer models, viz.1, 2-dimethyl hydrazine (DMH), and xenografts model. Protection to mice against benzo(a)pyrene [B(a)P] induced forestomach carcinogenesis and chromosomal aberrations (CAs) in bone marrow cells by TQ was reported by Badary *et al.* (1999). From their observation, tumor incidence and multiplicity was seen inhibited in as much as 70% and 67%, respectively. The growth inhibitory and antitumor effects of TQ were further studied by Badary and Gamal El Din (2001) in fibrosarcoma induced by 20-methylcholanthrene (MC) in male Swiss albino mice. TQ was found effective not only in significantly inhibiting tumor incidence and tumor burden (34% compared to 100% in control tumor-bearing mice), but it also delayed the onset of MC-induced fibrosarcoma tumors—indicative of chemopreventive action against MC-induced fibrosarcomas. Kaseb *et al.* (2007) observed in a xenograft prostate tumor model that TQ inhibited growth of C4–2B derived tumors in nude mice. This was associated with a dramatic decrease in androgen receptor, transcription factor E2F-1, and cyclin A as determined by Western blot analysis. Their findings clearly suggest that TQ may prove to be an effective agent in treating hormone sensitive, as well as hormone refractory, prostate cancers with reasonable degree of selectivity. TQ was also shown in another study of human prostate cancer (PC3 cells) xenograft to inhibit the tumor growth and block angiogenesis with almost no toxic side effects (Chehl *et al.*, 2009).

**TQ and Chemosensitization of Cancer**

Preclinical studies reveal the potential of TQ in improving the therapeutic effect of anticancer drugs and also protection of non tumor tissues against chemotherapy-induced damages. For example, TQ ameliorated nephrotoxicity and cardiotoxicity by cisplatin,
ifosfamide, and doxorubicin (Badary et al., 1997; Al-Shabanah et al., 1998; Badary et al., 2000). Of interest, Gali-Muhtasib et al. (2004) noted that TQ induces a sharp increase in p16 protein levels within 2 h of treatment; this observation is of interest, since as mentioned by Hochhauser, (1997) and Gali-Muhtasib et al. (2006), modulation of p16 protein expression increases tumor sensitivity to chemotherapeutic drugs. A study reported by Barron et al. (2008) evaluated proliferation of osteoblasts cells (MG 63) following a combined dose of TQ and selenium (Se). Their results revealed reduction in cell proliferation, increased cellular damage, decreased alkaline phosphatase levels, and decreased GST levels, indicating that the combined use of TQ and selenium (Se) may be an effective treatment option against human osteosarcoma cells (Barron et al., 2008). The chemosensitizing effect of TQ to conventional chemotherapeutic agents both in vitro and as well as in vivo were recently reported.

TQ has demonstrated its therapeutic effects in cancer and inflammation through different modes of action. This compound was found to be a potent free radical and superoxide radical scavenger, while preserving the activity of various antioxidant enzymes, such as catalase, glutathione peroxidase and glutathione-S-transferase. These effects were beneficial in various disease models, including experimental allergic encephalomyelitis, diabetes, asthma and carcinogenesis in animals. Numerous evidences have reported different modes of anticancer action, including anti-proliferation, cell cycle arrest, apoptosis induction, synergism with conventional medicine, ROS generation, and suppression of cancer metastasis and angiogenesis. Moreover, TQ could attenuate toxicity associated with the use of conventional medicine without compromising therapeutic efficacy. Present study was performed to have insight into effectiveness of TQ in attenuating the genotoxicity induced by iron.
### Quercetin Content
\( \text{mg/100gm edible portion} \)

<table>
<thead>
<tr>
<th>Food</th>
<th>Quercetin Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elderberries</td>
<td>42</td>
</tr>
<tr>
<td>Red Onions</td>
<td>33</td>
</tr>
<tr>
<td>White Onions</td>
<td>21</td>
</tr>
<tr>
<td>Cranberries</td>
<td>15</td>
</tr>
<tr>
<td>Green Hot Peppers</td>
<td>15</td>
</tr>
<tr>
<td>Kale</td>
<td>7.7</td>
</tr>
<tr>
<td>Blueberries</td>
<td>5.1</td>
</tr>
<tr>
<td>Red Apples</td>
<td>4.7</td>
</tr>
<tr>
<td>Romaine Lettuce</td>
<td>4.5</td>
</tr>
<tr>
<td>Pears</td>
<td>4.5</td>
</tr>
<tr>
<td>Spinach</td>
<td>4.1</td>
</tr>
</tbody>
</table>

### Antioxidant Potential of Quercetin
1.6.2. Quercetin

QCT (3,3',4',5,7-pentahydroxyflavone) belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plants and plant food sources. Frequently QCT occurs as glycosides (sugar derivatives); e.g., rutin in which the hydrogen of the R-4 hydroxyl group is replaced by a disaccharide. QCT is termed the aglycone, or sugarless form of rutin. Two extensive volumes, the proceedings of major meetings on plant flavonoids, presented much of the biological and medical data about QCT in 1985 and 1987 (Cody, 1986; 1988).

![Diagram of quercetin molecule]

Figure 11. 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one

1.6.2.1. Properties, Structure, Production and Occurrence

QCT is a yellow, crystalline solid with a bitter taste, which is insoluble in water, slightly soluble in alcohol, and soluble in glacial acetic acid and aqueous alkaline solutions (Weast et al., 1979; Windholz et al., 1983). QCT is a member of a group of naturally occurring compounds, the flavonoids, which have a common flavone nucleus composed of two benzene rings linked through a heterocyclic pyrone ring (Figure 11). Animals are unable to synthesize the flavones nucleus; thus, flavonoids are found exclusively in the plant kingdom. QCT and more than 2,000 other flavonoids occur as condensation products of p-glycosides (Herrmann, 1976; Kuhnau, 1976; Brown, 1980; IARC, 1983). QCT is found in various food products and plants, including fruits, seeds, vegetables, tea, coffee, bracken fern, and natural dyes (Table 2). QCT is usually obtained from the hydrolysis of rutin (QCT-3- rutinoside), a naturally occurring flavonoid glycoside (Griffith et al., 1955) although it can also be synthesized (Mack et al., 1962).
Table 2. QCT Content in Selected Foods

<table>
<thead>
<tr>
<th>Food Source</th>
<th>QCT Content (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple with skin</td>
<td>4.42</td>
</tr>
<tr>
<td>Broccoli, Raw</td>
<td>3.21</td>
</tr>
<tr>
<td>Raw Onions</td>
<td>13.27</td>
</tr>
<tr>
<td>Spinach, raw</td>
<td>4.28</td>
</tr>
<tr>
<td>Black Tea Leaves, dry</td>
<td>204.66</td>
</tr>
<tr>
<td>Green Tea Leaves, dry</td>
<td>255.55</td>
</tr>
<tr>
<td>Red Wine</td>
<td>0.84</td>
</tr>
</tbody>
</table>

1.6.2.2. Biosynthesis of QCT

The biosynthesis of phytochemicals, like flavonoids, is a defensive response of plants to their environment. Flavonoids often function as protection from ultraviolet sunlight and lipid peroxidation (Mariani et al., 2008). Mohle et al. (1985) demonstrated that when dill cell cultures were subjected to UV-B radiation, the predominant flavonoid synthesized was QCT-3-O-β-glucuronide. They proposed that the biosynthesis of flavonoids is regulated by ultraviolet light and their accumulation acts as a defense (Mohle et al., 1985).

1.6.2.3. Absorption, Metabolism, Distribution and Excretion

Absorption of QCT glycosides are relatively poor in small intestine. Micro floras of the lower bowel hydrolyze the flavonide-glycoside to QCT and the sugar, and QCT is then absorbed into the enterohepatic system (Brown, 1980; Tamura et al., 1980; Bokkenheuser et al., 1987). After oral administration of QCT to rabbits (Booth et al., 1956) or rats (Petrakis et al., 1959), three metabolites of QCT were identified in the urine: 3,4- dihydroxyphenylacetic acid, 3-methoxy-4-hydroxy phenylacetic acid (homovanillic acid), and m-hydroxyphenylacetic acid. These metabolites are thought to be formed in the liver after fusion of the ring. When Brown and Griffiths (1983)
administered QCT to rats by intraperitoneal injection, they identified the 3’o-methyl-ether of QCT (isorhamnetin) as a metabolite in bile (Brown and Griffiths, 1983).

The distribution, metabolism and excretion of 4-[14C] QCT in male ACI rats were studied by autoradiography and quantitation of radioactivity (Ueno et al., 1983). After oral administration, 20% of the dose was absorbed from the digestive tract and then excreted into the bile and urine within 48 hours as glucuronide or sulfate conjugates. Autoradiographic analysis of a rat 3 hours after receiving a single 2.3 mg/kg oral dose of QCT showed that most of the radioactivity remained in the digestive tract with low levels seen in the blood liver, kidney, lung, and rib. In five human volunteers, no QCT was detected in the plasma or urine after oral administration of 4g QCT (Gugler et al., 1975).

1.6.2.4. Mechanism of Action

1.6.2.4.1. Anti-oxidative action

The best described property of QCT is its ability to act as antioxidant (Figure 12). QCT seems to be the most powerful flavonoids for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or are induced by exogenous damage (Groot, 1994; Grace, 1994). One of the most important mechanisms and the sequence of events by which free radicals interfere with the cellular functions seem to be the lipid peroxidation leading eventually the cell death. To protect this cellular death to happen from reactive oxygen species, living organisms have developed antioxidant line of defense systems (Halliwell, 1995). These include enzymatic and non-enzymatic antioxidants that keep in check ROS/RNS level and repair oxidative cellular damage. The major enzymes, constituting the first line of defence, directly involved in the neutralization of ROS/RNS are: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The second line of defence is represented by radical scavenging antioxidants such as vitamin C, vitamin A and plant phytochemicals including QCT that inhibit the oxidation chain initiation and prevent chain propagation. This may also include the termination of a chain by the reaction of two radicals. The repair and de novo enzymes act as the third line of defence by repairing damage and reconstituting membranes. These include lipases, proteases, DNA repair enzymes and transferases (Bahorun et al., 2006).
1.6.2.4.2. Direct radical scavenging action

Free radical production in animal cells can either be accidental or deliberate. With the increasing acceptance of free radicals as common place and important biochemical intermediates, they have been implicated in a large number of human diseases (Ares and Outt, 1998; Wegener and Fintelmann, 1999). QCT acting as free radical scavengers was shown to exert a protective effect in reperfusion ischemic tissue damage (Fraga et al., 1987; Santos et al., 1998; Halliwell, 1994) (Figure 13).

1.6.2.4.3. Inducible nitric oxide syntheses Inhibitory action

QCT results in a reduction in ischemia-reperfusion injury by interfering with inducible nitric oxide synthase activity (Shoskes, 1998). QCT causes scavenging of free radicals; therefore can no longer react with nitric oxide, resulting in less damage (Shutenko et al., 1999). Nitric oxide interestingly can be viewed as radical itself and can directly be scavenged by Flavonoids (Van Acker et al., 1995).
1.6.2.4.4. Xanthine oxidase inhibitory action

The xanthine oxidase pathway has been implicated as an important route in the oxidative injury to the tissues especially after ischemia-reperfusion (Santrueza et al., 1992). Both xanthine dehydrogenase and xanthine oxidase are involved in the metabolism of xanthine to uric acid. Xanthine dehydrogenase is the form of the enzyme present under physiological condition but its configuration changed to xanthine oxidase during oxidative stress and ischemic conditions. QCT seems to inhibit xanthine oxidase activity thereby resulting in decreased oxidative injury (Iio, 1986; Chang, 1993; Shoskes, 1998).

1.6.2.5. Therapeutic Uses

QCT offers a variety of potential therapeutic uses primarily in the prevention and the treatment of the conditions listed below (Figure 14). QCT seems to work better when it is used in conjunction with bromelain, a digestive enzyme found in pineapple.
QCT might be useful in some of the allergies such as hay fever, hives. It inhibits the production and release of histamine and other allergic/inflammatory substances possibly by stabilizing cell membranes of mast cells (Lombard, 2005; Harnly et al., 2006). QCT seems to exert antibacterial activity against almost all the strains of bacteria known to cause respiratory, gastrointestinal, skin and urinary disorders (Rigano et al., 2006).

QCT inhibits both cyclo-oxygenase and lipo-oxygenase activities thus diminishing the formation of inflammatory mediators (Yoshimoto et al., 1983; Kim et al., 1998). In addition there are reports of people with rheumatoid arthritis, who experienced an improvement in their symptoms, when they switched from a typical western diet to a vegan diet with lots of uncooked berries, fruits, vegetables containing amongst other antioxidants, QCT (Hanninen, et al., 2000).

Although the etiology of cancer may be multifactorial (e.g. diet, genetic, environment), there is wide recognition that reactive oxygen and nitrogen species (ROS/RNS) play a pivotal role in the pathophysiological process. ROS/RON have been shown to be carcinogenic and may exert their deleterious effects by causing DNA damage, alter cell
signaling pathways (MAPK, NFkB, AP-1, PLA, ASK-1) and modulate gene expression (proto-oncogene, tumour suppressor gene). The evidence from *in vitro* and *in vivo* laboratory studies, clinical trials and epidemiological investigations show that plant-based diets have protective effects against various cancers. Indeed it has been suggested that about 7-31% of all cancers could be reduced by diets high in fruits and vegetables (Soo brattee *et al.*, 2006).

In a study done by Caltagirone *et al.*, (2000) QCT showed the inhibitory effect on the growth of melanoma and also influenced the invasive and metastatic potential in mice. The bioflavonoid QCT may be a potent alternative to reduce cisplatin induced nephrotoxicity (Heloisa *et al.*, 2004). Furthermore QCT seems to inhibit angiogenesis (Fotsis *et al.*, 1997). Angiogenesis is normally a strictly controlled process in the human body. Pathological, unregulated angiogenesis occurs in cancers (Fan *et al.*, 1995). Among the angiogenesis inhibitors QCT seems to play an important role (Paper, 1998).

Anti-oxidant QCT intake protects against coronary heart disease (CHD), caused by oxidized LDL (bad cholesterol). Hertog *et al.*, (1995) stated that regular consumption of flavonoids in the food might reduce the risk of deaths from CHD in elderly men (Hertog *et al.*, 1993; Hertog *et al.*, 1995). QCT was also shown to be effective inhibitor of platelets aggregation in dogs and monkeys (Osman *et al.*, 1998). The main antiplatelet aggregating effect is because of the inhibition of thromboxane A2 (Tzeng *et al.*, 1991).

QCT has been found to be an inhibitor of the enzyme aldose reductase, which plays a role in converting glucose (sugar) to sorbitol (a sugar alcohol) in the body. People with diabetes develop secondary problems, such as neuropathy, retinopathy, diabetic cataracts, and nephropathy because of sorbitol buildup in the body. QCT may therefore be beneficial in the nutritional management of diabetes, but clinical studies need to be conducted to verify these effects, which have been observed in non-human experiments (Costantino *et al.*, 1999).

Free radicals are thought to contribute the development of certain disorders including cataracts and macular degeneration. QCT prevents and treats these eye conditions by neutralizing these free radicals (Cai *et al.*, 2000). Regular consumption of dark berries offers benefits for preventing macular degeneration (Head, 1999). QCT by virtue of its
xanthine oxidase inhibitory nature prevents the production of uric acid, thereby easing the gout symptoms (Lio et al., 1986; Chang et al., 1993).

According to a study conducted by researchers at Cornell University in New York, a potent antioxidant (QCT) in apples and in vegetables appear to protect brain cells against oxidative stress, a tissue damaging process associated with Alzheimer and other neurodegenerative disorders (Heo et al., 2004). QCT seems to protect the brain functions by inhibiting the formation if fibrillated amyloid–beta, the senile plaque found in Alzheimer’s brain (Tzeng et al., 1991). An experiment was performed to demonstrate the possible effects of QCT on cognitive performance of young and aged, ethanol intoxicated mice (animal model), where chronic QCT treatment had shown the reversal of cognitive deficits (Singh et al., 2003). QCT has potential for the treatment of neuroleptic-induced extrapyramidal side effects, such as from haloperidol (Naidu and Kulkarni, 2004). QCT is also a powerful antioxidant that may protect brain cells from damage.

Hegarty et al., (2000) found that women, who drank tea (QCT), had higher bone mineral density measurements than those who did not drink tea. QCT in the tea might be responsible for the prevention of osteoporosis (). QCT seems to play a very important role in the prevention and treatment of peptic ulcer. QCT has been shown to inhibit the growth of (Helicobacter pylori) bacterium in in-vitro studies (Ailarcon de la Lastra et al., 1994; Martin et al., 1998).

In a prospective double-blind placebo controlled study, QCT was found to be helpful in category III chronic prostatitis (non bacterial chronic prostatitis and prostatodynia). Thirty men with this disorder received either placebo or 500 mg of QCT twice daily for one month. Significant improvement was achieved in treated group, as measured by the National Institute of Health Chronic Prostatitis score (Shoskes et al., 1999). The antiviral effect of flavonoid was shown in a study conducted by Wang et al. (1998). Some of the viruses reported to be affected by flavonoids are Herpes simplex virus, respiratory syncitial virus and adenovirus. QCT was reported to exhibit both anti-infective and antireplicative abilities. By far most of the studies were performed in vitro and little is known about the antiviral effect of flavonoids in vivo. There is some evidence that flavonoids in their glycon form seem to be more inhibitory effect on rotavirus infectivity than flavonoids in their aglycon form (Bae et al., 2000).
Flavonoids have received much attention due to their potential beneficial effects. QCT is the subject of intense research on the basis of its antioxidant, anti-inflammatory and anti-cancer activities. QCT and other flavonoids, have the potential to act as powerful antioxidants. QCT, being a major constituent of the flavonoid intake, could be a key in fighting several chronic degenerative diseases. However, most of the studies have been conducted in vitro; it is difficult to draw definite conclusion about the usefulness of flavonoids in the diet. Furthermore, insufficient methods are available to measure oxidative damage in vivo and the measurement of objective endpoints remains difficult. Although recently some studies (Aziz et al., 1998; McAnlis et al., 1999; Mullen et al.; 2006) have been conducted on absorption and excretion of flavonols including QCT but there is a need to improve analytic techniques to allow collection of more data in this aspect. The antioxidant activity of QCT’s metabolites and the pathways of metabolic conversion need to be identified and evaluated to accurately determine the effect of QCT in vivo and its effectiveness in preventing diseases arising from oxidative damage. Therefore, the present study was undertaken to have insight into effectiveness of QCT in ameliorating genotoxicity in vivo that will give a hopeful picture for the future.
Chapter 2
Objectives of the Study
OBJECTIVES

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 antimitogenic and anticarcinogenic activities. A systematic screening of plant extracts, food supplements or dietary products for their potential chemopreventive and antimitogenic activity 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 *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.
Chapter 3
Materials and Methods
MATERIALS AND METHODS

3.1. Test Animals
3.1.1. Species and Strains

Wistar rats were used in the present study. The choice was usually be determined by cost, availability and convenience.

3.1.2. Sex

The present study was carried out in male Wistar rats. Extensive studies of the activity of known clastogens in the mouse bone marrow micronucleus test have shown that in general male rats are more sensitive than female rats for micronucleus induction. A detailed collaborative study was carried out indicating that in general male rats were more sensitive than female rats for micronucleus induction, but where differences were seen they were only quantitative and not qualitative (The Collaborative Study Group for the Micronucleus Test, 1986). This analysis has been extended by the group considering the micronucleus test at the International Workshop on Standardisation of Genotoxicity Procedures, Melbourne, 1993 and having analysed data on 53 in vivo clastogens (and 48 non-clastogens), the same conclusions were drawn (Hayashi et al., 1984).

3.1.3. Weight

Adult male Wistar rats weighing 180-200 g b.w. were used in this study.

3.1.4. Age

There is generally a higher mitotic index in younger animals and so it is suggested that animals that have reached sexual maturity be used (e.g. 10-12 wk for rat), and that those of advanced age be avoided.

3.1.5. Housing

The animals were maintained on standard laboratory condition i.e.; room temperature of 18 ± 2°C; relative humidity 45-55%, 12:12h light/dark cycle and given food and water ad libitum. The animals were made to acclimatize with the laboratory conditions for a period of 15 days in a healthy condition prior to study. The whole experiment was
Chapter-3: Materials and Methods

designed as per Animal Ethical Committee Guidelines. All the protocols and the experiments were conducted in strict compliance with ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

3.1.6. Chemicals

Iron sulfate (CAS 7782-63-0), TQ (CAS 490-91-5), QCT (117-39-5), propidium iodide (P4170) (CAS 25535-16-4) and giemsa stain (CAS 51811-82-6) were procured from Sigma, USA. Other chemicals such as ethylene diamine tetraacetic acid (EDTA) disodium (054448), triton-X-100 (2020130), tris base (2044122), and 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, 75277) were purchased from SRL, India. Potassium chloride (Merck-7447-40-7), sodium chloride (Merck-7647-14-5), sodium hydroxide (Merck-1310-73-2), methanol (Merck-67-56-1), glacial acetic acid (Merck-64-19-7), sodium bicarbonate (Merck-144-55-8), low melting point (LMP) agarose (Merck-9012-36-6), glycerol (Merck-56-81-5), colchicines (Merck-64-86-8), May-Grünwald’s eosine-methylene blue solution modified (200-659-6) and xylene (1330-20-7) were purchased from Merck Pvt ltd.

3.2. Chemical treatments

3.2.1. Route of administration

Intraperitoneal injection is the most commonly used method of administration largely for simplicity, and because, for many agents, it will tend to maximize chemical exposures to the bone marrow. However, this is not always the case, and other exposure routes can be equally or more effective. The route of administration of test compound in the present study was oral and intraperitoneal both.

3.2.2. Solvent or vehicle

Ferrous sulfate is water-soluble chemical so it was most desirable to dissolve the compound in water because exposure was oral for this chemical. The other chemicals like TQ and QCT were not soluble in water so were dissolved in organic solvents such as dimethyl sulfoxide (DMSO). The injection volume was kept as small as feasible i.e. accurate delivery was maintained. For chemicals in saline or water, volumes of 0.2-0.4 ml are recommended, but a maximum of 0.1 ml is necessary when DMSO is the solvent.
3.2.3. Dose selection

The doses of iron sulfate were selected on the basis of its effectiveness in inducing chromosomal aberrations and on the basis of available data (Benoni et al., 1993). Doses of TQ 6, 9, 12, 15, 18, 21 and 24 mg/kg b.w. were selected on the basis of available data that exhibited antimutagenic effects (Kaseb et al., 2007; Gali-Muhtasib et al., 2008). The doses of QCT (125, 250, 375, 500, 625, 750 and 875 mg/kg) were also determined on the basis of available data that exhibited antimutagenic effects (Da Silva et al., 2002; Leelayuwat et al., 2012).

3.2.4. Negative Control

A group of animals was included as control for the solvent (DMSO) used. The volume of solvent injected and the route of exposure were the same as that used for the groups treated with the test compounds.

3.2.5. Positive Control

Positive controls are included to establish the ability of analyzers to correctly ascertain aberrations, to determine the expected variations from test to test and animal to animal within an assay, and perhaps most importantly to establish the sensitivity of a particular test. Cyclophosphamide 25 mg/kg i.p. was used as a positive control in the present study.

3.2.6. Number of animals and administration of doses

The number of animals to be used for each group was large 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 blood cells of rats. The rats were separated randomly into 5 groups of 6 animals each. The first group was used as control and administered with 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
mgFe/kg. The rats were separated randomly into 9 groups of 6 animals each. Group 1
was used as control and administered with DMSO and 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 QCT 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 control
and administered with DMSO and Group 2 was administered FeSO₄ at the dose of 200
mgFe/kg and groups 3-9 were administered pre, simultaneous and post treatment of QCT
at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg with the administration of
FeSO₄ 200 mg Fe/kg.

3.3. Mammalian Bone Marrow Chromosomal Aberration Assay
3.3.1. Principles

The assay is based on the ability of a test agent to induce chromosome structural or
numerical alterations that can be visualized microscopically. The target tissue for the
chromosomal aberration assay is the bone marrow because it is a rapidly dividing, well
vascularized tissue. A minimum of 5 rodents (mice, rats or Chinese hamsters) per sex per
group are administered the test chemical, preferably only once, by an appropriate route
of exposure, typically by gavage or intraperitoneal injection. Three doses are prepared so
that they span a range from the maximum tolerable dose to a dose level that does not
induce appreciable toxicity. The maximum tolerable dose should be identified in a
preliminary test as that producing toxicity such that higher doses would be expected to
lead to mortality, or the dose producing evidence of bone marrow cytotoxicity (i.e.,
>50% reduction of the mitotic index).

Following administration, sampling should usually be conducted twice. In order to
accumulate metaphase cells, cell division is arrested by administration of a mitotic
inhibitor, such as colchicine, 3-5 hours prior to sacrifice. Cells in their first metaphase
after administration should be examined, so that any induced increase in the frequency of
chromosomal aberrations can be related to the treatment. Therefore, half of the animals
should be sacrificed for the first sampling after a time period equivalent to 1.5x the normal cell cycle length (usually 12-18 hours for most species used). Because of the potential for some chemicals to induce mitotic delay, the remainder of the animals should be sacrificed 24 h after the first sampling time. Bone marrow cells are obtained immediately, exposed to hypotonic solution, fixed and stained. Using light microscopy, an observer should score a minimum of 1000 cells/animal for mitotic index and a minimum of 100 metaphase cells/animal for chromosomal aberrations. Cells scored for chromosomal aberrations should have a number of centromeres within the range 2n ± 2, because chromosomes can be lost during slide preparation. A test chemical that induces an increase in the frequency of structural aberrations, including chromosome-type and chromatid-type aberrations is considered to be clastogenic under the test conditions. A test chemical that induces an alteration in the frequency of aneuploid cells is considered to be aneugenetic. There are two types of chromosomal aberrations as described below:

3.3.2. Sampling times and slide preparation

3.3.2.1. Sampling time

Rats were sacrificed after 24 h of each treatment by using ether as anesthesia. All procedures were carried out according to the international practices for animal use and care. In order to accumulate metaphase cells, and provide more readily analyzable chromosomes a mitotic inhibitor (Colchicine- 2 mg/kg body weight) was injected i.p. usually 2 h before killing the animals.

3.3.2.2. Slide preparation, Sacrifice and harvest

Femurs were quickly removed, muscle was cleaned from the bone and both femurs were placed on the edge of a prenumbered plastic centrifuge tube which corresponds to the animal number. The cells in bone marrow were flushed with a hypodermic syringe fitted with a 22-g needle by flushing 0.075 M KCl 2-3 times into the marrow cavity of femur until after careful observation no bone marrow remains attached to bone. After a few seconds to allow fragments to settle, the suspension was decanted to the other corresponding tube. The tubes were centrifuged for about 10 min at 1000 rpm. Supernatant was removed by gentle aspiration unit a small volume remains above the pellet. The pellet was resuspended in the remaining volume.
3.3.2.3. Hypotonic treatment

0.075 M KCl (prewarmed to 37°C) was added drop wise with agitation to approximately 5 ml. It was incubated for 20 min in 37°C water bath and centrifuged at 1,000 rpm for 10 min. The supernatant was removed. The pellet was resuspended in the remaining volume.

3.3.2.4. Fixation

0.5 ml fixative (3:1 absolute methanol: glacial acetic acid made freshly immediately before using) was added drop wise using a Pasteur pipette while continuously shaking the pellet so as to avoid formation of clots. It was allowed to stand at room temperature for 15-20 min. In order to ensure proper fixation, the cells were kept suspended in the fixative at 4°C for a minimum period of 1-h but preferably overnight. The contents, then, were again centrifuged at 1000 rpm for 10 minutes and the same two or three changes with fresh fixative were given before preparing the slide.

3.3.2.5. Slide preparation

After giving final washing in the fixative, the cells were resuspended in 0.2 ml of fresh fixative. Slides were prepared by flame drying technique. Slides were washed clean and immediately coded and kept at 4°C in distilled water. About 3-4 drops of cell suspension were dropped per slide with the help of Pasteur’s pipette over the chilled, tilted slides maintaining a stretched hand distance between the slides and the pipette to get better spreading, and a brief exposure to the flame was given to dry the slides. Three drops/slide was generally enough to get a satisfactory cell count. The slides were kept in a slide box under dust free conditions for further use. After one-hour slides were stained in 5% Giemsa stain in phosphate buffer (pH 6.8).

3.3.2.6. Scoring and Photography

At least 100 well-spread metaphases have to be scored per animal of each treatment and control. The experimental unit is the cell, and therefore the numbers of cells with structural chromosome aberrations have to be evaluated. Detectable and finely spread metaphase chromosome spots were analyzed under 100X oil immersion, position of the desired spot was marked by reading scales over the microscope. Photographs were taken with an automatic digital camera attached in a Nikon microscope 80i.
3.4. Micronucleus Assay

3.4.1. Principles

The micronucleus assay detects chromosome damage and whole chromosome loss in polychromatric erythrocytes, and eventually in normochromatic erythrocytes in peripheral blood as the red cells mature. A micronucleus is a small structure (1/5 to 1/20 the size of the nucleus) containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. Micronuclei can be found in cells of any tissue, but only form in dividing cells. There are four generally accepted mechanisms through which micronuclei can form: a) the mitotic loss of acentric chromosome fragments (forming structural aberrations), b) mechanical consequences of chromosomal breakage and exchange, such as from lagging chromosomes, an inactive centromere or tangled chromosomes (forming structural aberrations), c) mitotic loss of whole chromosomes (forming numerical aberrations) and, d) apoptosis. However, nuclear fragments resulting from apoptosis are usually easy to identify because they are much more numerous or pyknotic than those induced by clastogenic or aneugenic mechanisms. Structural aberrations are believed to result from direct or indirect interaction of the test chemical with DNA, while numerical aberrations are often a result of interference with the mitotic apparatus preventing normal nuclear division.

Bone marrow is the major haematopoietic tissue in the adult rodent. Administration of a chemical during proliferation of haematopoietic cells may cause chromosome damage or inhibition of the mitotic apparatus. These chromosome fragments or whole chromosomes may lag behind during cell division and form micronuclei. The erythrocyte is particularly well suited to analysis for micronuclei because during maturation of the erythroblast to the polychromatric erythrocyte (a period of about 6 hours following the final mitosis), the nucleus is extruded, making detection of micronuclei easier. In addition, the polychromatric erythrocyte (PCE) still contains RNA, and so it stains blue-grey with Giemsa or reddish with acridine orange. This allows differentiation from mature, haemoglobin-containing erythrocytes (NCE), which stain orange with Giemsa or are unstained by acridine orange, and facilitates identification of the cells where micronuclei induced by the test substance may be present. Sampling of PCEs from the bone marrow or peripheral blood prior to their differentiation to mature erythrocytes is critical; once a
PCE has matured, associating the presence of micronuclei in these cells with acute chemical exposure is not possible. Mature erythrocytes persist in peripheral circulation for about 1 month.

The micronucleus assay is conducted using the bone marrow or peripheral blood of rodents, typically mice, as the target tissue; the peripheral blood of species other than the mouse can be used if there is evidence that demonstrates micronucleated erythrocytes are not rapidly removed by the spleen. The test chemical is administered to a minimum of five animals of each sex per group orally by gavage or by intraperitoneal injection. Three doses are prepared so that the dose range spans a range from the maximum tolerable dose to a dose level that does not induce appreciable toxicity. One of two treatment schedules is recommended: the test chemical is administered once and one group is sacrificed at 24 h and another at 48 hours after treatment (for bone marrow), or at 36 and 72 hours after treatment (for peripheral blood); if multiple treatments are used, a single sample can be taken between 18-24 h after the last administration for bone marrow or between 36-48 hours for peripheral blood. A delay between chemical administration and sampling of PCEs is necessary to allow sufficient time for the number of micronucleated PCEs to rise to a peak. This corresponds to the time necessary for absorption and metabolism of the chemical, the completion of the erythroblast cell cycle, including any test chemical-induced cell-cycle delay, and for extrusion of the erythroblast nucleus. Because the incidence of micronucleated PCEs is rare in untreated animals, to allow for appropriate statistical power, at least 2000 PCEs per animal should be scored for the incidence of micronuclei. The proportion of PCE among total erythrocytes should also be determined from at least 200 total erythrocytes for bone marrow samples or from at least 1000 for peripheral blood samples. Data indicating the test chemical depressed the PCE: total erythrocyte (TE) ratio are suggestive of cytotoxicity and provide evidence that the test chemical reached the bone marrow. However, significant clinical symptoms of toxicity or plasma test chemical concentrations may also be used as a measure of exposure, since the bone marrow is a relatively well-perfused tissue and the plasma test substance concentration should approximate the level of bone marrow exposure. However, if the test chemical is severely cytotoxic, finding and scoring 2000 PCEs at higher concentrations may be difficult; in these circumstances it is particularly important to have multiple dose groups. Because micronuclei are relatively rare, manual enumeration
by light microscopy is time consuming. For that reason, newer flow cytometric or image analysis methods have been adapted for the rapid processing of slides. These methods have yet to undergo a thorough evaluation, but they offer tremendous potential for improving the sensitivity and the efficiency of the assay. Any test chemical that induces an increase in the frequency of micronucleated PCE is concluded to have induced chromosomal aberrations in vivo, but further mechanistic information useful to distinguish micronuclei induced by clastogenic or aneugenic chemicals can be obtained. Micronuclei of aneugenic origin will contain centromeres, the presence of which can be verified using one of two molecular cytogenetic methods: immunofluorescent CREST-staining or fluorescence in situ hybridization (FISH) with pancentromeric DNA probes. This information is useful in risk assessment because aneugens may exhibit threshold dose-responses that are not typical of those chemicals that interact directly with DNA.

3.4.2. Sampling times and slide preparation

3.4.2.1. Sampling time

Rats were sacrificed after 24 h of each treatment by using ether as anesthesia. All procedures were carried out according to the international practices for animal use and care.

3.4.2.2. Extraction of bone marrow from animals

This assay was performed according to the method described by Schmid (1975). Before sacrifice the animals, a 5-ml centrifuge tube is filled with fetal calf serum for each individual. This admittedly expensive fluid has proved superior to all other rinsing solutions or cheaper sera tried so far. With substitutes the cells were often damaged or the erythrocytes were partially agglutinated resulting in preparations that were unusable or consumed too much time for analysis. From a single rat one can easily obtain enough bone marrow cells for several slides. From the freshly killed animal both femora are removed in toto. The bones are then freed from muscle by the use of gauze and fingeis. The proximal end of the femur is carefully shortened with scissors until a small opening to the marrow canal becomes visible. With the needle of appropriate size mounted, about 0.2 ml serum is pulled from the tube into a disposable plastic syringe. Then the needle is inserted a few mm into the proximal part of the marrow canal which is still closed at the distal end. Next, the femur is submerged completely in the serum and squeezed against
the tube to prevent the bone from slipping off the needle. Subsequently, the marrow is aspirated; should the needle have become obstructed, the serum in the syringe is first pressed out. After several gentle aspirations and flushings, the process is repeated from the distal end of the femur. The bone marrow cells should get into the serum as a fine suspension and not in the form of gross particles.

3.4.2.3. Preparation of the smears

The tube is centrifuged at 1000 rpm for 5 min. The supernatant is removed with a Pasteur pipette. If the sediment is large, half a drop of serum is left; if it is minute, all the supernatant is drawn off. The cells in the sediment are carefully mixed by aspiration into the capillary part of a fresh, siliconized Pasteur pipette. A small drop of the viscous suspension is put on the end of a slide and spread by pulling the material behind a polished cover glass held at an angle of 45 degrees. The size of the droplet is chosen so that all material is used up at a distance of 2-3 cm. The preparations are then air dried.

3.4.2.4. Staining

The best results are obtained if staining takes place the day following preparation. If staining is done immediately, the slides must be flamed shortly. Staining is carried out in ordinary vertical staining jars according to the following procedure: staining for 3 min in undiluted May-Gruenwald solution; staining for 2 more min in May-Gruenwald diluted with distilled water 1: 1; staining for 10 min in Giemsa diluted with distilled water 1: 6; rinsed in distilled water; blotted dried with filter paper; cleaned back side of slide with methanol; cleared in xylene for 5 min, and mount with cover glass.

3.4.2.5. Analyzing the slides

First the slides are screened, at medium magnification, for regions of suitable technical quality, where the cells are well spread, undamaged and perfectly stained. Such regions are normally located in a zone close to the end of the smear. A perfect morphology of the nucleated cells serves as criterion for good quality, even though the nucleated cells are not evaluated in the test. The erythrocytes must be well spread, neither globular nor having slurred contours. Their staining has to be vigorous, red in mature erythrocytes (anulocytes) and with a strong bluish tint in the immature forms (polychromatic erythrocytes). For each animal, three slides were prepared and the best slide was selected
for scoring. The incidences of micronuclei (MN), observed in 2000 PCE/rat were calculated to find the clastogenic property of the test chemicals.

3.5. Single cell gel electrophoresis (SCGE/Comet assay)

3.5.1. Principle of Comet assay

In the Comet assay, the cells are embedded in a thin agarose gel on a microscope slide. The cells are then lysed to remove all cellular proteins and the DNA is allowed to unwind under alkaline/neutral conditions. Following unwinding the DNA is electrophoresed and stained with a fluorescent dye. During electrophoresis, broken DNA fragments (damaged DNA) or relaxed chromatin migrates away from the nucleus. The extent of DNA liberated from the head of the comet was directly proportional to the DNA damage. There are many possible explanations on how comet assay works. Many evidence points to the fact that during the lysis step, the membranes, cytoplasm and nucleoplasm are removed leaving a halo of DNA embedded in agarose. DNA damage induces relaxation in the super-coiled DNA coils, which are then pulled to one side by electrophoresis. According to this theory, a single strand break should uncoil and relax the DNA, which can then be picked up on the Comet assay. There are also evidences suggesting that DNA damage induces small fragments of DNA which are then separated on electrophoresis. Comet assay essentially measures the degree of relaxation as well as fragmentation of DNA within the cell. It is therefore necessary to convert DNA damage into strand breaks that relax the super-coiled DNA or produce DNA fragments before detected on Comet assay. The simplest types of DNA damage detected by Comet assay are the Double Strand Breaks (DSBs). DSBs within the DNA results in DNA fragments and can be detected by merely subjecting them to electrophoretic mobility at the neutral pH. Single Strand Breaks (SSBs) does not produce DNA fragments unless the two strands of the DNA are separated/denatured. This is accomplished by unwinding the DNA at pH 12.1. It is also possible that single strand breaks can relax the DNA and hence can also be detected on Comet assay at a neutral pH. Other types of DNA damage broadly termed as alkali labile sites (ALS) are expressed when the DNA is treated with alkali at pH greater than pH13. Furthermore, breaks can be introduced at the sites of DNA base modifications by treating the DNA with lesion-specific glycosylases/ endonucleases and the fragments thus produced can also be detected by Comet assay. Therefore, by controlling the conditions that produces nicks at the sites of specific DNA
lesions, Comet assay can be used to detect various classes of DNA damage. While breaks increase DNA migration, DNA binding and Crosslinks (DNA-DNA or DNA-Protein Interactions) crosslinks can retard DNA migrations, and can also be detected on Comet assay.

**Definition of some important comet parameters**

**Tail Length**: Tail Length is the distance of DNA migration from the body of the nuclear core and it is used to evaluate the extent of DNA damage.

**Olive Tail Moment**: Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). Olive Tail moment = (Tail mean - Head mean) X Tail%DNA/100.

**Extent Tail Moment** = (Tail Length x Tail % DNA)/ 100.

**Head % DNA** = [Head.Opt-Inten/ (Head.Opt-Inten + Tail.Opt-Inten)] x 100.

**Tail % DNA** = 100 – Head % DNA.

3.5.2. Sampling times and slide preparation

3.5.2.1. Sampling time

Rats were sacrificed 24 h after each treatment by using ether as anesthesia. All procedures were carried out according to the international practices for animal use and care.

3.5.2.2. Blood collection

Blood samples were collected by cardiac puncture using a 21-gauge needle and syringe, and immediately transferred into tubes containing sterile EDTA solution (to a final concentration of 1.6 mg EDTA/ml of blood). Blood was processed immediately for comet assay.

3.5.2.3. Slide preparation

This assay was performed in dark according to the method described by Buschini et al. (2002) with slight modifications. 0.8% low melting point (LMP) agarose was prepared in
saline and was maintained at 39°C to prevent solidification. Subsequently, 20μl of whole blood treated with iron sulfate alone and/or iron sulfate along with different concentrations of TQ and QCT were gently mixed with 250μl of 0.8% LMP agarose. The resulting suspension was layered onto the frosted side of fully frosted slides. The slides were placed on ice for approximately 5 min to allow the agarose to solidify.

3.5.2.4. Lysis

Once prepared, slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA with fresh 1% Triton-X-100 and 10% DMSO) for 1 h to eliminate non-nuclear components prior to the alkali unwinding step.

3.5.2.5. Unwinding and electrophoresis

The slides were further immersed in alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min to allow the DNA to unwind and to convert alkali labile sites to single strand breaks. Electrophoresis was conducted for 30 min at 15 V and 200 mA (at a rate of 0.6 V/cm) using a compact power supply.

3.5.2.6. Neutralization and dehydration of slides

After completion of electrophoresis, the slides were gently washed with 0.4 M Tris (pH = 7.5) to remove alkali and detergents for at least 10 minutes.

3.5.2.7. DNA staining, comet visualization and analysis

The slides were placed in a humid chamber until staining to prevent the gel from drying. The cells were stained with propidium iodide (PI – 20 μg/ml) and were observed under a fluorescent microscope. Images of the cells were obtained using a digital camera. Approximately 60–80 images per slide were captured from different imaging fields and were analyzed with the appropriate software. For each image, the two SCGE parameters, including the olive tail moment (OTM) and tail moment (TM) were analyzed. Olive tail moment = (tail mean –head mean) × tail%DNA/100; tail moment is = tail length × tail%DNA (tail intensity)/100.
Chapter 4
Results
RESULTS

ACUTE TOXICITY OF IRON SULFATE

4.1.1. Evaluation of acute toxicity of iron sulfate by chromosomal aberration assay in bone marrow cells of Wistar rats for 24 h.

Structural aberrations (chromatid and chromosome types) induced by different treatments were enumerated in the present study, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings. All these types of structural abnormalities and their frequencies for both control and treated groups are presented in Table 1. The mean number of aberrant cells was found to be 124±18.03, 1.83 ±0.09, 34.33±7.13, 59.83±10.62 and 110.83±13.56 for positive control, negative control, 50 mgFe/kg, 100 mgFe/kg and 200 mgFe/kg of iron sulfate respectively (Table 1, Figure 1 and 2).

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. The animals treated with iron sulfate at the dose of 200 mgFe/kg had a statistically significant increment in the number of chromosomal aberrations (P < 0.001) when compared with the negative control.
Table 1. Chromosomal aberrations in Wistar rat bone marrow cells treated with iron sulfate and respective control for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells (mg/kg body weight) Studied/n</th>
<th>Dose</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control (CP)</td>
<td>600/6</td>
<td>25 mg/kg</td>
<td>48.5±20.13</td>
<td>33.1±8.06</td>
<td>17.1±7.12</td>
<td>7.8±2.98</td>
<td>17.3±4.98</td>
<td>124±18.03**</td>
</tr>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>---</td>
<td>0.5±0.89</td>
<td>0.3±0.25</td>
<td>0.3±0.31</td>
<td>0.1±0.11</td>
<td>0.5±0.14</td>
<td>1.8±0.09</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>50 mg Fe/kg</td>
<td>12.1±5.83</td>
<td>7.6±2.12</td>
<td>6.3±3.43</td>
<td>2.1±1.03</td>
<td>4.3±1.92</td>
<td>34.3±7.13**</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>100 mg Fe/kg</td>
<td>23.1±8.72</td>
<td>18.6±4.87</td>
<td>10.0±4.85</td>
<td>2.5±1.04</td>
<td>5.5±1.63</td>
<td>59.8±10.62**</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>200 mg Fe/kg</td>
<td>46.1±10.32</td>
<td>31.1±9.25</td>
<td>12.1±5.61</td>
<td>5.1±2.83</td>
<td>16.1±5.72</td>
<td>110.1±13.50**</td>
</tr>
</tbody>
</table>

One hundred cells were analyzed per animal, for a total of 600 cells per treatment. Significantly different from the control *P < 0.05; **P < 0.001, CP= cyclophosphamide, FeSO₄= iron sulfate, X= mean, SD= standard deviation.
Figure 1. Graphical representation of bone marrow cells containing chromosome aberrations after treatment with increasing doses of FeSO₄ for 24 h.

Male Wistar rats received FeSO₄ at the doses of 50, 100 and 200 mg Fe/kg. Data are expressed as the mean ±SD. The level of significance was set at $P<0.05$. Statistically significantly different compared to control: *$P<0.05$; **$P<0.001$. NC= normal control; CP= cyclophosphamide; FeSO₄= iron sulfate; SD= standard deviation.
Figure 2. Photomicrographs illustrating the effects of iron sulfate (FeSO₄) on bone marrow cells of Wistar rats (A) Chromatid break; (B) Ring chromosome, Acentric chromosome; (C) Chromatid break; (D) Chromosome break and Tri radial formation.
4.1.2. Assessment of acute toxicity of iron sulfate by micronucleus assay in bone marrow cells for 24 h.

The MN data for rat bone marrow cells following treatment with iron sulfate are presented in Table 2. The number of MNPCEs among 2000 PCEs is an indicative of genotoxicity. The mean number of MNPCEs was found to be 40.83+3.69, 2.16+0.99, 8.16+2.38, 18.66+4.55 and 30.16+5.37 for positive control, negative control, 50 mgFe/kg, 100 mgFe/kg and 200 mgFe/kg of iron sulfate respectively (Table 2, Figure 3 and 4).

No statistically significant increase in the mean number of MNPCEs was in animals treated with iron sulfate at the dose of 50 and 100 mgFe/kg in comparison to the 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.
Table 2. Iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h</th>
<th>Dose (mg/kg body weight)</th>
<th>MNPCEs per 2000 Polychromatic Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PCE/n</td>
<td></td>
<td></td>
<td>X±SD</td>
</tr>
<tr>
<td>Positive control (CP)</td>
<td>12000/6</td>
<td>25 mg/kg</td>
<td>(50, 44, 38, 39, 46, 28)</td>
</tr>
<tr>
<td>Negative control</td>
<td>12000/6</td>
<td>---</td>
<td>(1, 2, 2, 1, 3, 4)</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>12000/6</td>
<td>50 mg Fe/kg</td>
<td>(9, 8, 6, 9, 7, 10)</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>12000/6</td>
<td>100 mg/kg</td>
<td>(25, 16, 21, 18, 13, 19)</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>12000/6</td>
<td>200 mg/kg</td>
<td>(32, 24, 31, 26, 40, 28)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of 6 Wistar rats in each group and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Turkey post hoc test was used to examine the differences between samples. P values difference between the ten groups of rat bone marrow MNPCEs results statistically significant different compared to control: * P < 0.05; ** P < 0.001 and CP= cyclophosphamide, FeSO₄= iron sulfate, PCE= polychromatic erythrocyte, MNPCEs= micronucleated polychromatic erythrocytes, X= mean, SD= Standard deviation.
Figure 3. Mean number of micronucleated polychromatic erythrocytes (MNPCeEs) in 2000 polychromatic erythrocytes (PCEs) in animals treated with increasing doses of FeSO₄ for 24 h.

Male Wistar rats received FeSO₄ at the doses of 50, 100 and 200 mg Fe/kg. Data are expressed as the mean ±SD. The level of significance was set at $P < 0.05$. Statistically significantly different compared to control: * $P < 0.05$; ** $P < 0.001$. NC= normal control; CP= cyclophosphamide; FeSO₄= iron sulfate; SD= standard deviation.
Figure 4. Representative images of polychromatic erythrocytes (PCEs) containing micronuclei in bone marrow smear of Wistar rats after 24 h of iron sulfate treatment. [A, B, C, D, F, G, H, I] micronucleus in polychromatic (immature) erythrocyte, bluish in colour. [E]: micronucleus in normochromic (matured) erythrocytes.
4.1.3. Assessment of acute toxicity of iron sulfate by single cell gel electrophoresis (SCGE/comet assay)

The effect of the iron sulfate treatment on the extent of DNA damage is presented in Table 3 as mean values of tail moment and olive tail moment for each rat. Using CASP software we evaluated parameters for measuring DNA damage. To describe the comet, the two SCGE parameters, including olive tail moment (OTM) and tail moment (TM) were analyzed. The results of the alkaline Comet assay give the mean values of tail moment (Figure 5a) and olive tail moment (Figure 5b) for the treated and control groups. The mean value of DNA damage in terms of tail moment was found to be 42.2±3.9, 1.23±0.78, 6.33±2.76, 18.51±5.45 and 33.78±4.56 for positive control, negative control, 50 mgFe/kg, 100 mgFe/kg and 200 mgFe/kg of iron sulfate respectively (Table 3, Figure 3a) and the mean value of DNA damage in terms of olive tail moment was found to be 46.4±3.21, 1.99±0.89, 9.74±3.18, 22.63±5.83 and 38.98±6.51 for positive control, negative control, 50 mgFe/kg, 100 mgFe/kg and 200 mgFe/kg of iron sulfate respectively (Table 3, Figure 3b). Treatment by iron sulfate at the dose of 50 and 100mgFe/kg did not induce any significant increase in mean tail moment and olive tail moment. However, significantly higher levels of DNA damage were detected in rats exposed to iron sulfate at the dose of 200 mgFe/kg when compared with the negative control (Table 3, Figure 5). In summary, iron sulfate induced dose dependent significant increase in DNA damage in exposed rats (Table 3 and Figure 5a, b and 6).
Chapter 4: Results

Table 3. Mean and standard deviation of comet tail moment and olive tail moment in whole blood of Wistar rats treated with iron sulfate for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h</th>
<th>Dose</th>
<th>Tail moment (TM)</th>
<th>Olive tail moment (OTM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cells/n</td>
<td>(mg/kg body weight)</td>
<td>(X±SD)</td>
<td>(X±SD)</td>
</tr>
<tr>
<td>Positive control (CP)</td>
<td>300/6</td>
<td>25 mg/kg</td>
<td>42.2±3.9</td>
<td>46.4±3.21**</td>
</tr>
<tr>
<td>Negative control</td>
<td>300/6</td>
<td>---</td>
<td>1.23±0.78</td>
<td>1.99±0.89</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>50 mg Fe/kg</td>
<td>6.33±2.76</td>
<td>9.74±3.18*</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>100 mg Fe/kg</td>
<td>18.51±5.45</td>
<td>22.63±5.83**</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>200 mg Fe/kg</td>
<td>33.78±4.56</td>
<td>38.98±6.51**</td>
</tr>
</tbody>
</table>

Measures were calculated for 50 cells per rats (six rats per group). Values are expressed as mean ± SD and were analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. Statistically significant different compared to control: *P < 0.05; **P < 0.001. CP = cyclophosphamide, FeSO₄ = iron sulfate, X = mean, SD = standard deviation.
Figure 5a. DNA damage induced by FeSO₄ in bone marrow cells after treatment with increasing doses of FeSO₄ for 24 h assessed through comet parameter: Tail moment.

Male Wistar rats received FeSO₄ at the doses of 50, 100 and 200 mg Fe/kg. Data are expressed as the mean ±SD. The level of significance was set at $P<0.05$. Statistically significantly different compared to control: $* P < 0.05$; $** P < 0.001$. NC= normal control; CP= cyclophosphamide; FeSO₄= iron sulfate; SD= standard deviation.

Figure 5b. DNA damage induced by FeSO₄ in bone marrow cells after treatment with increasing doses of FeSO₄ for 24 h assessed through comet parameter: Olive tail moment.

Male Wistar rats received FeSO₄ at the doses of 50, 100 and 200 mg Fe/kg. Data are expressed as the mean ±SD. The level of significance was set at $P<0.05$. Statistically significantly different compared to control: $* P < 0.05$; $** P < 0.001$. NC= normal control; CP= cyclophosphamide; FeSO₄= iron sulfate; SD= standard deviation.
Figure 6. SCGE (Comet) pictures illustrating the effects of iron sulfate ($\text{FeSO}_4$) on the extent of DNA damage. [A] Control (DMSO); [B] Positive Control (CP); [C] treated with $\text{FeSO}_4$ at the dose of 50 mgFe/kg; [D] treated with $\text{FeSO}_4$ at the dose of 100 mgFe/kg; [E] treated with $\text{FeSO}_4$ at the dose of 200 mgFe/kg, respectively.
PROTECTIVE EFFECT OF THYMOQUINONE

4.2.1. Protective effect of TQ on iron sulfate induced chromosome aberrations

4.2.1.1. Pre-Treatment with TQ

The types of chromosome aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by iron sulfate (200 mg/kg) alone as well as pre-treatment with the different doses of TQ are presented in Table 4. The mean number of aberrant cells was found to be 2.0±0.89, 104.17±11.14, 98.17±9.28, 92.00±14.05, 86.67±10.80, 77.33±9.09, 71.33±5.95, 74.83±7.57 and 82.17±12.46 for negative control, treatment with 200 mgFe/kg iron sulfate alone and treatment with iron sulfate (200 mgFe/kg) along with TQ pre-treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg respectively (Table 4, Figure 7 and 10). Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the number of 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 chromosome aberrations between the control and the animals treated with iron sulfate and TQ pre-treatment (P < 0.001). In the presence of TQ (6, 9, 12, 15, 18, 21 and 24 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). The ameliorating effect of TQ on iron sulfate induced chromosome aberrations was most prominent at the dose of 18 mg/kg.

4.2.1.2. Optimum level of TQ

The broken-line regression analysis of the TQ levels against Chromosomal aberrations induced by FeSO₄ exhibited reduction in chromosomal aberrations and maximum reduction was observed at 18 mg/kg of TQ during pre-treatment against chromosomal aberrations induced by iron sulfate. The equations and optimum level of TQ are as under,

\[ Y = 111.48333 - 6.63, \quad X \leq 18 \text{ mg/kg} \quad (r^2=0.99696); \quad Y = 38.17 + 5.42, \quad X \geq 18 \text{ mg/kg} \]

\[ (r^2= 0.97972) \quad X_{opt} = 18 \text{ mg/kg TQ (Pre-treatment with TQ)} \]

The equations for TQ doses to chromosomal aberrations induced by FeSO₄ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect and is given in the respective figure (Figure 7).
### Chapter 4: Results

Table 4. The apportionment of structural aberrations observed in bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of TQ pretreatment.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h</th>
<th>Dose (mg/kg body weight)</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>---</td>
<td>0.66±0.51</td>
<td>0.50±0.54</td>
<td>0.33±0.51</td>
<td>0.00±0.00</td>
<td>0.50±0.54</td>
<td>2.0±0.89</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>200 mg Fe/kg</td>
<td>43.33±6.47</td>
<td>32.50±2.88</td>
<td>12.00±3.68</td>
<td>5.00±2.09</td>
<td>11.33±3.82</td>
<td>104.17±11.14**</td>
</tr>
<tr>
<td>FeSO₄+TQ₁</td>
<td>600/6</td>
<td>6 mg/kg</td>
<td>40.50±9.26</td>
<td>31.16±11.92</td>
<td>11.33±3.55</td>
<td>4.66±2.33</td>
<td>10.50±4.63</td>
<td>98.17±9.28*</td>
</tr>
<tr>
<td>FeSO₄+TQ₂</td>
<td>600/6</td>
<td>9 mg/kg</td>
<td>38.00±8.07</td>
<td>29.66±4.45</td>
<td>10.16±4.95</td>
<td>4.33±2.80</td>
<td>9.83±4.75</td>
<td>92.00±14.05**</td>
</tr>
<tr>
<td>FeSO₄+TQ₃</td>
<td>600/6</td>
<td>12 mg/kg</td>
<td>36.50±9.97</td>
<td>28.16±12.00</td>
<td>9.33±4.76</td>
<td>4.00±2.96</td>
<td>8.66±3.82</td>
<td>86.67±10.80**</td>
</tr>
<tr>
<td>FeSO₄+TQ₄</td>
<td>600/6</td>
<td>15 mg/kg</td>
<td>33.83±15.60</td>
<td>26.33±10.32</td>
<td>7.83±5.49</td>
<td>2.66±1.63</td>
<td>6.66±3.44</td>
<td>77.33±9.09**</td>
</tr>
<tr>
<td>FeSO₄+TQ₅</td>
<td>600/6</td>
<td>18 mg/kg</td>
<td>32.00±9.75</td>
<td>24.83±8.47</td>
<td>7.16±3.60</td>
<td>1.83±1.47</td>
<td>5.50±3.39</td>
<td>71.33±5.95**</td>
</tr>
<tr>
<td>FeSO₄+TQ₆</td>
<td>600/6</td>
<td>21 mg/kg</td>
<td>33.00±9.12</td>
<td>25.33±7.96</td>
<td>7.50±3.98</td>
<td>2.50±1.87</td>
<td>6.50±3.50</td>
<td>74.83±7.57**</td>
</tr>
<tr>
<td>FeSO₄+TQ₇</td>
<td>600/6</td>
<td>24 mg/kg</td>
<td>35.33±8.77</td>
<td>26.83±6.73</td>
<td>8.33±4.88</td>
<td>3.50±1.51</td>
<td>8.16±3.37</td>
<td>82.17±12.46**</td>
</tr>
</tbody>
</table>

About 600 cells were analyzed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. X= mean; TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation. Statistically significantly different compared to control: * P<0.05; ** P<0.001. Statistically significantly different from FeSO₄ groups: (a) P<0.05; (b) P<0.005; (c) P<0.001.
Figure 7: Broken-line relationship of pre-treatment of TQ levels with FeSO₄ to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

Male Wistar rats received FeSO₄ alone at the dose of 200 mg Fe/kg and received TQ at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg 1 h before administration of FeSO₄ at 200 mg Fe/kg. Data are expressed as the mean ±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. The level of significance was set at *P<0.05. Broken-line regression analysis was employed to determine the optimum level of TQ. Statistically significantly different compared to control: * P<0.05; ** P<0.001. Statistically significantly different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.005; (c) P< 0.001. TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.

4.2.1.3. Simultaneous Treatment with TQ

The types of chromosome aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by iron sulfate (200 mg/kg) alone as well as simultaneous treatment with the different doses of TQ are presented in Table 5. The mean number of aberrant cells was found to be 2.16±1.16, 102.83±10.60, 83.83±11.58, 76.16±13.30, 65.83±6.46, 56.0±12.53, 41.0±5.95, 51.5±8.42 and 68.83±8.32 for negative control, treatment with 200 mgFe/kg iron sulfate alone and treatment with iron sulfate (200 mgFe/kg) along with TQ pre-treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg respectively (Table 5, Figure 8 and 10). Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the number of 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 chromosome aberrations between the control and the animals treated with iron sulfate and TQ simultaneous treatment (P<0.001). In the presence of TQ (6, 9, 12, 15, 18, 21 and 24 mg/kg), the
number of chromosom al 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). The greatest reduction of chromosomal aberrations was observed at the dose of 18 mg/kg. The ameliorating effect of TQ on iron sulfate induced chromosome aberrations was most prominent in simultaneous treatment.

4.2.1.4. Optimum level of TQ

The broken-line regression analysis of the protective dose of TQ levels against chromosomal aberrations induced by FeSO₄ exhibited reduction in chromosomal aberrations and maximum reduction was observed at 18 mg/kg of TQ during simultaneous treatment against chromosomal aberrations induced by iron sulfate. The equations and optimum level of TQ are as under,

\[
Y = 111.23867 - 11.51343, \quad X \leq 18 \text{ mg/kg } (r^2 = 0.99226); \quad Y = -43.62833 + 13.915, \quad X \geq 18 \text{ mg/kg}
\]

\[
(r^2 = 0.99011) \quad X_{opt} = 18 \text{ mg/kg TQ (Simultaneous treatment with TQ)}
\]

The equations for TQ doses to chromosomal aberrations induced by FeSO₄ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figure (Figure 8).
Chapter-4: Results

Table 5. The apportionment of structural aberrations observed in bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of TQ simultaneous treatment.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells Studied/u</th>
<th>Dose (mg/kg body weight)</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>---</td>
<td>0.83±0.40</td>
<td>0.50±0.54</td>
<td>0.16±0.40</td>
<td>0.16±0.40</td>
<td>0.50±0.54</td>
<td>2.16±1.16</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>200 mg Fe/kg</td>
<td>44.5±9.87</td>
<td>28.50±6.80</td>
<td>13.50±3.44</td>
<td>4.66±2.16</td>
<td>11.66±3.93</td>
<td>102.83±10.60**</td>
</tr>
<tr>
<td>FeSO₄+TQ₂</td>
<td>600/6</td>
<td>6 mg/kg</td>
<td>38.83±6.46</td>
<td>25.66±5.81</td>
<td>10.00±3.09</td>
<td>3.66±2.16</td>
<td>5.66±2.16</td>
<td>83.83±11.58*(a)</td>
</tr>
<tr>
<td>FeSO₄+TQ₃</td>
<td>600/6</td>
<td>9 mg/kg</td>
<td>36.0±6.08</td>
<td>23.5±6.18</td>
<td>8.83±2.31</td>
<td>2.66±1.63</td>
<td>5.16±2.40</td>
<td>76.16±13.30**(a)</td>
</tr>
<tr>
<td>FeSO₄+TQ₄</td>
<td>600/6</td>
<td>12 mg/kg</td>
<td>32.0±8.95</td>
<td>19.66±5.91</td>
<td>7.16±2.31</td>
<td>2.33±1.36</td>
<td>4.66±2.63</td>
<td>65.83±6.46**</td>
</tr>
<tr>
<td>FeSO₄+TQ₅</td>
<td>600/6</td>
<td>15 mg/kg</td>
<td>26.83±5.85</td>
<td>16.00±4.47</td>
<td>7.00±2.60</td>
<td>1.66±1.64</td>
<td>4.50±2.58</td>
<td>56.0±12.53**</td>
</tr>
<tr>
<td>FeSO₄+TQ₆</td>
<td>600/6</td>
<td>18 mg/kg</td>
<td>15.50±3.61</td>
<td>14.35±6.55</td>
<td>5.66±3.26</td>
<td>1.83±1.16</td>
<td>3.66±1.63</td>
<td>41.0±5.95**</td>
</tr>
<tr>
<td>FeSO₄+TQ₇</td>
<td>600/6</td>
<td>21 mg/kg</td>
<td>18.83±2.99</td>
<td>18.16±5.68</td>
<td>8.00±3.34</td>
<td>2.33±1.03</td>
<td>4.16±1.94</td>
<td>51.5±8.42**</td>
</tr>
</tbody>
</table>

About 600 cells were analysed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples.

X= mean; TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.
Statistically significant different compared to control: * P<0.05; ** P<0.001.
Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001.
Figure 8. Broken-line relationship of simultaneous treatment of TQ levels with FeSO₄ to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

Male Wistar rats received FeSO₄ alone at the dose of 200 mg Fe/kg and received simultaneous treatment of TQ at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg with FeSO₄ at 200 mg Fe/kg. Data are expressed as the mean ±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. The level of significance was set at $P<0.05$. Broken-line regression analysis was employed to determine the optimum level of TQ. Statistically significantly different compared to control: * $P<0.05$; ** $P<0.001$. Statistically significantly different from FeSO₄ groups: (a) $P<0.05$; (b) $P<0.001$. TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation

4.2.1.5. Post Treatment with TQ

The types of chromosome aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by iron sulfate (200 mg/kg) alone as well as post treatment with the different doses of TQ are presented in Table 6. The mean number of aberrant cells was found to be 1.16 ±0.75, 110.00±16.44, 100.50±23.72, 96.33±18.73, 85.00±23.96, 79.83±32.06, 71.83±18.88, 80.50±15.24 and 92.66±23.17 for negative control, treatment with 200 mgFe/kg iron sulfate alone and treatment with iron sulfate (200 mgFe/kg) along with TQ post treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg respectively (Table 6, Figure 9 and 10). Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the number of 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 chromosome aberrations between the control and the animals treated with iron sulfate and TQ post treatment ($P<0.001$). In the presence of TQ (6, 9, 12, 15,18, 21 and 24 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). The ameliorating effect of TQ on iron sulfate induced chromosome aberrations was most prominent at the dose of 18 mg/kg.

4.2.1.6. Optimum level of TQ

The broken-line regression analysis of the TQ levels against chromosomal aberrations induced by FeSO$_4$ exhibited reduction in chromosomal aberrations and maximum reduction was observed at 18 mg/kg of TQ during post treatment against chromosomal aberrations induced by iron sulfate. The equations and optimum level of TQ are as under,

$$Y = 117.00067 - 7.54829, \quad X \leq 18 \text{ mg/kg} \quad (r^2 = 0.99513); \quad Y = 8.75833 + 10.415, \quad X \geq 18 \text{ mg/kg}$$

$(r^2 = 0.99535) \quad X_{\text{opt}} = 18 \text{ mg/kg} \text{ TQ (Post treatment with TQ)}$

The equations for TQ doses to chromosomal aberrations induced by FeSO$_4$ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figure (Figure 9).
Table 6. The apportionment of structural aberrations observed in bone marrow cells of Wistar rats 24 h after single administration of iron sulfate with post treatment of TQ.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells Studied/ n</th>
<th>Dose (mg/kg body weight)</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>---</td>
<td>0.33±0.51</td>
<td>0.33±0.51</td>
<td>0.16±0.40</td>
<td>0.16±0.40</td>
<td>0.16±0.40</td>
<td>1.16±0.75</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>200 mg Fe/kg</td>
<td>42.16±16.43</td>
<td>33.83±10.16</td>
<td>16.00±5.05</td>
<td>4.83±1.72</td>
<td>13.16±3.65</td>
<td>110.00±16.44**</td>
</tr>
<tr>
<td>FeSO₄+TQ₁</td>
<td>600/6</td>
<td>6 mg/kg</td>
<td>38.83±8.23</td>
<td>31.83±7.78</td>
<td>14.50±4.67</td>
<td>4.16±2.13</td>
<td>11.16±4.95</td>
<td>100.50±23.72*(a)</td>
</tr>
<tr>
<td>FeSO₄+TQ₂</td>
<td>600/6</td>
<td>9 mg/kg</td>
<td>37.83±10.14</td>
<td>30.66±8.14</td>
<td>13.50±4.08</td>
<td>4.33±2.58</td>
<td>10.00±3.34</td>
<td>96.33±18.73*(a)</td>
</tr>
<tr>
<td>FeSO₄+TQ₃</td>
<td>600/6</td>
<td>12 mg/kg</td>
<td>34.83±9.08</td>
<td>28.50±7.76</td>
<td>12.16±7.02</td>
<td>3.83±1.47</td>
<td>5.66±1.96</td>
<td>85.00±23.96**(a)</td>
</tr>
<tr>
<td>FeSO₄+TQ₄</td>
<td>600/6</td>
<td>15 mg/kg</td>
<td>33.16±12.02</td>
<td>27.33±7.11</td>
<td>10.83±4.16</td>
<td>3.50±1.87</td>
<td>6.33±3.07</td>
<td>79.83±32.06**(b)</td>
</tr>
<tr>
<td>FeSO₄+TQ₅</td>
<td>600/6</td>
<td>18 mg/kg</td>
<td>30.00±6.92</td>
<td>24.00±7.82</td>
<td>9.50±3.01</td>
<td>3.16±1.60</td>
<td>5.16±2.13</td>
<td>71.83±18.88**(b)</td>
</tr>
<tr>
<td>FeSO₄+TQ₆</td>
<td>600/6</td>
<td>21 mg/kg</td>
<td>32.00±11.66</td>
<td>29.00±7.69</td>
<td>10.50±4.18</td>
<td>3.50±1.76</td>
<td>5.50±2.42</td>
<td>80.50±15.24**(a)</td>
</tr>
<tr>
<td>FeSO₄+TQ₇</td>
<td>600/6</td>
<td>24 mg/kg</td>
<td>34.66±9.54</td>
<td>32.00±10.23</td>
<td>14.50±7.06</td>
<td>4.00±1.78</td>
<td>7.50±3.01</td>
<td>92.66±23.17**(a)</td>
</tr>
</tbody>
</table>

About 600 cells were analysed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. X= mean; TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.

Statistically significant different compared to control: * P< 0.05; ** P< 0.001.

Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001.
Figure 9. Broken-line relationship of post treatment of TQ levels with FeSO₄ to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

Male Wistar rats received FeSO₄ alone at the dose of 200 mg Fe/kg and received post treatment of TQ at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg with FeSO₄ at 200 mg Fe/kg. Data are expressed as the mean ±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. The level of significance was set at $P<0.05$. Broken-line regression analysis was employed to determine the optimum level of TQ. Statistically significantly different compared to control: * $P<0.05$; ** $P<0.001$. Statistically significantly different from FeSO₄ groups: (a) $P<0.05$; (b) $P<0.001$. TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.
Figure 10. Photomicrographs illustrating the effects of iron sulfate (FeSO₄) and thymoquinone (TQ) on bone marrow cells of Wistar rats (A) Chromatid break; (B) Dicentric chromosome; (C) Ring chromosome; (D) Acentric fragment.
4.2.2. Protective Effects of TQ on Micronucleus Induced By Iron Sulfate 200 mgFe/kg

4.2.2.1. Pre-Treatment with TQ

The number of MNPCEs among 2000 PCE, indicative of genotoxicity, is presented in Table 7. The mean value of MNPCEs was found to be 1.66 ± 0.81, 29.66±7.81, 26.33±6.23, 21.5±6.17, 19.33±4.33, 15.5±2.98, 11.33±3.05, 14.16±2.66 and 18.5±4.10 for negative control, 200 mgFe/kg iron sulfate alone, cells treated with iron sulfate (200 mgFe/kg) and pre-treatment with 6, 9, 12, 15, 18, 21 and 24 mg/kg doses of TQ respectively (Table 7, Figure 11 and 14). Iron sulfate induced a significant ($P<0.001$) increase in MNPCEs number at a dose of 200 mg/kg. TQ at the doses of 6, 9, 12, 15 and 18 mg/kg, induced statistically significant decreases in the mean value of MNPCEs induced by iron sulfate in pre-treatments. TQ showed significant inhibitory effect on iron sulfate induced micronuclei in PCE. All doses of TQ tested were found to be effective in reducing the mean value of MNPCEs induced by iron sulfate except 21 and 24 mg/kg doses. With iron sulfate the lower doses of TQ were not effective in reducing the mean value of MNPCEs. The most effectively significant inhibitory effect on MN formation in PCE ($P<0.001$) was observed at 18 mg/kg dose of TQ. The protective influence of TQ on iron sulfate induced in the MNPCEs was most prominent at 18 mg/kg (Figure 11).

4.2.2.2. Optimum level of TQ

The broken-line regression analysis of the TQ levels against MNPCEs induced by FeSO₄ exhibited reduction in this parameter was best attained at 18 mg/kg of TQ during pre-treatment against genotoxicity induced by iron sulfate. The equations and optimum level of TQ are as under,

\[ Y = 33.24444 - 3.60952X \leq 18 \text{ mg/kg (} r^2 = 0.99701\); \quad Y = 10.41667 + 3.58333, \quad X \geq 18 \text{ mg/kg} \]

\[ (r^2 = 0.99278) X_{opt} = 18 \text{ mg/kg TQ (Pre-treatment with TQ)} \]

The equations for TQ doses to MNPCEs induced by FeSO₄ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figure (Figure 11).
4.2.2.3. Simultaneous Treatment with TQ

The number of MNPCES among 2000 PCE, indicative of genotoxicity, is presented in Table 7. The mean value of MNPCES was found to be 1.33 ± 0.86, 30.0±9.62, 25.5±6.65, 22.5±3.22, 17.16±2.84, 13.66±2.64, 8.16±1.78, 12.5±2.31 and 17.83±3.77 for negative control, 200 mgFe/kg iron sulfate alone, cells treated with iron sulfate (200 mgFe/kg) and simultaneous treatment with 6, 9, 12, 15, 18, 21 and 24 mg/kg doses of TQ respectively (Table 7, Figure 12 and 14). Iron sulfate induced a significant (P<0.001) increase in MNPCES number at a dose of 200 mg/kg. TQ at the doses of 6, 9, 12, 15 and 18 mg/kg, induced statistically significant decreases in the mean value of MNPCES induced by iron sulfate in simultaneous treatments. TQ showed significant inhibitory effect on iron sulfate induced micronuclei in PCE. All doses of TQ tested were found to be effective in reducing the mean value of MNPCES induced by iron sulfate except 21 and 24 mg/kg doses. With iron sulfate the lower doses of TQ were not effective in reducing the mean value of MNPCES. The most effectively significant inhibitory effect on MN formation in PCE (P<0.001) was observed at 18 mg/kg dose of TQ. The protective influence of TQ on iron sulfate induced in the mean value of MNPCES was most prominent in the simultaneous treatments (Figure 12).

4.2.2.4. Optimum level of TQ

The broken-line regression analysis of the TQ levels against MNPCES induced by FeSO₄ exhibited that the reduction in this parameter was best attained at 18 mg/kg of TQ during simultaneous treatment against genotoxicity induced by iron sulfate. The equations and optimum level of TQ are as under,

\[ Y = 34.5 - 4.28571X \text{, } X \leq 18 \text{ mg/kg} \ (r^2 = 0.99731); \ Y = -21 + 4.83333X \ \text{, } X \geq 18 \text{ mg/kg} \]

\( (r^2 = 0.99822) \ X_{opt} = 18 \text{ mg/kg TQ (Simultaneous treatment with TQ)} \)

The equations for TQ doses to MNPCES induced by FeSO₄ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figure (Figure 12).
4.2.2.5. Post Treatment with TQ

The number of MNPCES among 2000 PCE, indicative of genotoxicity, is presented in Table 7. The mean value of MNPCES was found to be 2.16 ± 1.09, 29.33±8.86, 25.66±7.76, 21.5±4.55, 20.33±3.78, 16.5±5.34, 13.16±3.44, 15.83±4.01 and 19.0±6.71 for negative control, 200 mgFe/kg iron sulfate alone, cells treated with iron sulfate (200 mgFe/kg) and post treatment with 6, 9, 12, 15, 18, 21 and 24 mg/kg doses of TQ respectively (Table 7 Figure 13 and 14). Iron sulfate induced a significant (P<0.001) increase in MNPCES number at a dose of 200 mg/kg. TQ at the doses of 6, 9, 12, 15 and 18 mg/kg, induced statistically significant decreases in the mean value of MNPCES induced by iron sulfate in post treatments. TQ showed significant inhibitory effect on iron sulfate induced micronuclei in PCE. All doses of TQ tested were found to be effective in reducing the mean value of MNPCES induced by iron sulfate except 21 and 24 mg/kg doses. With iron sulfate the lower doses of TQ were not effective in reducing the mean value of MNPCES. The most effectively significant inhibitory effect on MN formation in PCE (P< 0.001) was observed at 18 mg/kg dose of TQ. The protective influence of TQ on iron sulfate induced in the MNPCES was most prominent at 18 mg/kg (Figure 13).

4.2.2.6. Optimum level of TQ

The broken-line regression analysis of the TQ levels against MNPCES induced by FeSO₄ exhibited reduction in this parameter was best attained at 18 mg/kg of TQ during post treatment against genotoxicity induced by iron sulfate. The equations and optimum level of TQ are as under,

\[ Y= \frac{32.03333-3.12857}{X \leq 18 \text{ mg/kg} \ (r^2=0.99365)}; \quad Y=\frac{-4.41667+2.91667}{X \geq 18 \text{ mg/kg}} \]

\( (r^2=0.99878) \ X_{\text{opt}} = 18 \text{ mg/kg TQ (Post treatment with TQ)} \)

The equations for TQ doses to MNPCES induced by FeSO₄ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figure (Figure 13).
<table>
<thead>
<tr>
<th>Group</th>
<th>X+SD</th>
<th>X+SD</th>
<th>X+SD</th>
<th>X+SD</th>
<th>Post Treatment with TQ</th>
<th>TQ Treatment with TQ</th>
<th>Total PCEC (mg/kg body weight)</th>
<th>A24 L Per 2000 Potyphomeric Proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Pre-simultaneous and post-treatment of TQ-mediated modification of iron sulphone induced micromolecules in bone marrow cells of Wistar rats.

Further analysis using one-way analysis of variance (ANOVA) for multiple comparisons.

Values are expressed as mean ± SD of 6 Wistar rats in each group and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons.

**Note:**
- **p < 0.001:** strongly significant difference compared to control.
- **p < 0.05:** significantly higher than their respective controls.
Figure 13

Figure 12

Figure 11

Induced monocytes in ploychretic erythrocytes (PECs) in bone marrow cells of visceral fats.

Figure 4: Brooks' Relationship of PI, simultaneous and post treatment with T0 levels to reduction in iron sulfate
Figure 14. Representative images of polychromatic erythrocytes (PCEs) containing micronuclei in bone marrow smear of Wistar rats after 24 h of iron sulfate treatment along with different doses of thymoquinone. [A, B, C, D, E, G, H, I] micronucleus in polychromatic (immature) erythrocyte, bluish in colour. [F]: micronucleus in normochromatic (matured) erythrocytes.
4.2.3. Protective Effect of TQ on DNA Damage Induced By Iron Sulfate 200 mgFe/kg

4.2.3.1. Pre treatment with TQ

The effects of iron sulfate and TQ treatment on the extent of DNA damage are presented in Table (8) as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. To describe the comet, two SCGE parameters, including tail moment (TM) and olive tail moment (OTM) were analyzed.

**Tail moment (TM)**

The mean value of DNA damage in terms of TM in control rats was 0.79±0.65. The mean value of comet TM measured in rats treated with iron sulfate was 25.38±4.30 which was significantly higher ($P < 0.001$) in comparison to control groups. Interestingly, the TM induced by iron sulfate 200 mg Fe/kg was reduced by TQ pretreatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg which were 1.46 ± 0.75, 31.33±10.23, 24.3±8.67, 20.54±9.43, 17.88±5.62, 10.98±3.12, 6.48±1.67, 9.29±2.33 and 14.66±3.31 respectively, and the most significant decrease in DNA damage in terms of decreased TM was 6.48±1.67 at 18 mg/kg dose of TQ in comparison to control (Table 8 and Figure 15a).

**Olive tail moment (OTM)**

The mean value of DNA damage in terms of OTM in control rats was a comet OTM measured in rats treated with iron sulfate was with a mean value 29.38±4.12 which was significantly higher ($P < 0.001$) in comparison to control groups. The OTM induced by iron sulfate 200 mg Fe/kg was reduced by TQ pretreatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg which were 1.96 ± 1.02, 38.13±11.31, 31.34±9.55, 22.87±8.53, 20.09±5.69, 14.23±3.76, 8.23±2.71, 13.78±4.10 and 18.21±4.29 respectively and significant decrease in DNA damage in terms of decreased OTM was observed 8.23±2.71 at 18 mg/kg dose of TQ in comparison to iron sulfate treated groups (Table 8 and Figure 15b).

Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a dose of 200 mg/kg compared with the negative
control ($P < 0.001$). Statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the control and cells treated with iron sulfate and TQ in the pre-treatments (except 21 and 24 mg/kg dose of TQ $P < 0.001$). In the presence of TQ doses (6, 9, 12, 15 and 18 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P < 0.001$. The protective influence of TQ on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in 18 mg/kg (Figure 15 a,b and 16).

4.2.3.2. Optimum level of TQ

The broken-line regression analysis of pre-treatment with TQ levels against DNA damage induced by FeSO$_4$ exhibited reduction in this parameter was best attained at 18 mg/kg of TQ. The equations and optimum level of TQ are as under,

\[ Y = 35.272-4.76771, \quad X \leq 18 \text{ mg/kg} \quad (r^2=0.99256); \quad Y = -18.48667+4.09, \quad X \geq 18 \text{ mg/kg} \]

\[ (r^2=0.98407) \quad X_{\text{opt}} = 18 \text{ mg/kg} \quad \text{TQ (Pre-treatment with TQ) (TM)} \]

\[ Y = 42.84267-5.81743, \quad X \leq 18 \text{ mg/kg} \quad (r^2=0.99317); \quad Y = -21.52333+4.99, \quad X \geq 18 \text{ mg/kg} \]

\[ (r^2=0.99791) \quad X_{\text{opt}} = 18 \text{ mg/kg} \quad \text{TQ (Pre-treatment with TQ) (OTM)} \]

The equations for TQ doses to DNA damage induced by FeSO$_4$ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figures (Figure 15 a,b).
Table 8. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone and pretreatment in combination with different doses of TQ for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>Pre-treatment with TQ</th>
<th>At 24 h</th>
<th>Dose (mg/kg body weight)</th>
<th>Tail moment (TM) (X±SD)</th>
<th>Olive tail moment (OTM) (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>300/6</td>
<td>--</td>
<td>1.46 ± 0.75</td>
<td>1.96 ± 1.02</td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>200 mg Fe/kg</td>
<td>31.33±10.23**</td>
<td>38.13±11.31**</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₀</td>
<td>300/6</td>
<td>6 mg/kg</td>
<td>24.3±6.67**(a)</td>
<td>31.34±9.55**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₁</td>
<td>300/6</td>
<td>9 mg/kg</td>
<td>20.5±9.43**(a)</td>
<td>22.8±8.53**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₂</td>
<td>300/6</td>
<td>12 mg/kg</td>
<td>17.8±5.62**(a)</td>
<td>20.0±5.69**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₃</td>
<td>300/6</td>
<td>15 mg/kg</td>
<td>10.9±3.12**(a)</td>
<td>14.2±3.76**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₄</td>
<td>300/6</td>
<td>18 mg/kg</td>
<td>6.4±1.67**(b)</td>
<td>8.2±2.71**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₅</td>
<td>300/6</td>
<td>21 mg/kg</td>
<td>9.2±2.33**(b)</td>
<td>13.7±4.10**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₆</td>
<td>300/6</td>
<td>24 mg/kg</td>
<td>14.6±3.31**(a)</td>
<td>18.2±4.29**(a)</td>
<td></td>
</tr>
</tbody>
</table>

Measures were calculated for 50 cells per rat (six rats per group). Values are expressed as mean ± SD and were analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. Statistically significant different compared to control: * P<0.05; ** P<0.001 and statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.001. FeSO₄=iron sulfate, TQ=thymoquinone, X=mean, SD=standard deviation.
Figure 15 a. Broken-line relationship of pre-treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of TQ in whole blood cells of Wistar rats assessed through comet parameter: tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001. Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.

Figure 15 b. Broken-line relationship of pre-treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of TQ in whole blood cells of Wistar rats assessed through comet parameter: Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001. Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.
Figure 16. SCGE (Comet) pictures illustrating the effects of iron sulfate and thymoquinone (TQ) pre-treatment on the extent of DNA damage. [A] Control (DMSO); [B] treated with FeSO₄ at the dose of 200 mg Fe/kg; [C, D, E, F, G, H, I] treated with TQ at the dose of 6, 9, 12, 15, 18, 21 and 24 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
4.2.3.3. Simultaneous treatment with TQ

The effects of iron sulfate and TQ treatment on the extent of DNA damage are presented in Table (9) as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. To describe the comet, two SCGE parameters, including tail moment (TM) and olive tail moment (OTM) were analyzed.

**Tail moment (TM)**

The mean value of DNA damage in terms of TM in control rats was 0.79±0.65. The mean value of comet TM measured in rats treated with iron sulfate was 25.38±4.30 which was significantly higher ($P < 0.001$) in comparison to control groups. Interestingly, the TM induced by iron sulfate 200 mg Fe/kg was reduced by TQ simultaneous treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg which were 0.93±0.51, 29.38±9.54, 22.24±9.23, 18.37±5.44, 14.76±6.98, 8.19±2.13, 4.05±1.25, 6.99±1.89 and 12.54±3.04 respectively, and the most significant decrease in DNA damage in terms of decreased TM was 4.05±1.25 at 18 mg/kg dose of TQ in comparison with control (Table 9 and Figure 17a).

**Olive tail moment (OTM)**

The mean value of DNA damage in terms of OTM in control rats was 0.90±0.87. The comet OTM measured in rats treated with iron sulfate was with a mean value 29.38±4.12 which was significantly higher ($P < 0.001$) in comparison to control groups. The OTM induced by iron sulfate 200 mg Fe/kg was reduced by TQ simultaneous treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg which were 1.88±0.96, 35.67±10.06, 28.34±7.34, 22.81±5.61, 17.66±4.32, 14.23±5.23, 7.54±3.24, 9.39±1.56 and 13.44±2.39 respectively and significant decrease in DNA damage in terms of decreased OTM was observed 7.54±3.24 at 18 mg/kg dose of TQ in comparison to iron sulfate treated groups (Table 9 and Figure 17b).

Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a dose of 200 mg/kg compared with the negative control ($P < 0.001$). Statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the control and cells treated with
iron sulfate and TQ in the simultaneous treatment (except 21 and 24 mg/kg dose of TQ ($P < 0.001$). In the presence of TQ doses (6, 9, 12, 15 and 18 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P < 0.001$. The protective influence of TQ on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in simultaneous treatment (Figure 17 a, b and 18).

4.2.3.4. Optimum level of TQ

The broken-line regression analysis of the simultaneous treatment with TQ levels against DNA damage induced by FeSO$_4$ data exhibited that the reduction in this parameter was best attained at 18 mg/kg of TQ. The equations and optimum level of TQ are as under,

\[ Y = 33.406 - 4.926, \quad X \leq 18 \text{ mg/kg} \quad (r^2 = 0.9952); \quad Y = -21.855 + 4.245, \quad X \geq 18 \text{ mg/kg} \]

\[ r^2 = 0.98461 \quad X_{\text{opt}} = 18 \text{ mg/kg TQ (Simultaneous treatment with TQ) (TM)} \]

\[ Y = 39.85467 - 5.37514, \quad X \leq 18 \text{ mg/kg} \quad (r^2 = 0.99519); \quad Y = -10.52667 + 2.95, \quad X \geq 18 \text{ mg/kg} \]

\[ r^2 = 0.9776 \quad X_{\text{opt}} = 18 \text{ mg/kg TQ (Simultaneous treatment with TQ) (OTM)} \]

The equations for TQ doses to DNA damage induced by FeSO$_4$ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figures (Figure 17 a, b).
Table 9. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone and simultaneous treatment in combination with different doses of TQ for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>Simultaneous treatment with TQ</th>
<th>At 24 h</th>
<th>Dose</th>
<th>Tail moment (TM)</th>
<th>Olive tail moment (OTM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total cells</td>
<td>(mg/kg body weight)</td>
<td>(X±SD)</td>
<td>(X±SD)</td>
</tr>
<tr>
<td>Negative control</td>
<td>30/0/6</td>
<td>---</td>
<td>0.93 ± 0.51</td>
<td>1.38 ± 0.96</td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
<td>30/0/6</td>
<td>200 mg Fe/kg</td>
<td>29.2±9.34**</td>
<td>35.67±10.06**</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₁</td>
<td>30/0/6</td>
<td>6 mg/kg</td>
<td>22.2±9.23**(a)</td>
<td>28.3±7.04**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₂</td>
<td>30/0/6</td>
<td>9 mg/kg</td>
<td>18.3±5.44**(a)</td>
<td>22.8±5.61**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₃</td>
<td>30/0/6</td>
<td>12 mg/kg</td>
<td>14.7±6.98**(a)</td>
<td>17.6±4.32**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₄</td>
<td>30/0/6</td>
<td>15 mg/kg</td>
<td>8.19±2.13**(a)</td>
<td>14.2±5.23**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₅</td>
<td>30/0/6</td>
<td>18 mg/kg</td>
<td>4.05±1.25**(b)</td>
<td>7.5±4.23**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₆</td>
<td>30/0/6</td>
<td>21 mg/kg</td>
<td>6.99±1.89**(b)</td>
<td>9.3±1.56**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + TQ₇</td>
<td>30/0/6</td>
<td>24 mg/kg</td>
<td>12.5±4.04**(b)</td>
<td>13.4±4.39**(b)</td>
<td></td>
</tr>
</tbody>
</table>

Measures were calculated for 50 cells per rat (six rats per group). Values are expressed as mean ± SD and were analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons. Turkey post hoc test was used to examine the differences between samples. Statistically significant different compared to control: * P< 0.05; ** P< 0.001 and statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001. FeSO₄=iron sulfate, TQ=thymoquinone, X=mean, SD=standard deviation.
Figure 17a. Broken-line relationship of simultaneous treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of TQ in whole blood cells of Wistar rats assessed through comet parameter: tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P < 0.05; ** P < 0.001, Statistically significant different from FeSO₄ groups: (a) P < 0.05; (b) P < 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.

Figure 17b: Broken-line relationship of simultaneous treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of TQ in whole blood cells of Wistar rats assessed through comet parameter: Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P < 0.05; ** P < 0.001, Statistically significant different from FeSO₄ groups: (a) P < 0.05; (b) P < 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.
Figure 18. SCGE (Comet) pictures illustrating the effects of iron sulfate and thymoquinone (TQ) simultaneous treatment on the extent of DNA damage. [A] control (DMSO); [B] treated with FeSO₄ at the dose of 200 mg Fe/kg; [C, D, E, F, G, H, I] treated with TQ at the dose of 6, 9, 12, 15, 18, 21 and 24 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
4.2.3.5. Post Treatment with TQ

The effects of iron sulfate and TQ treatment on the extent of DNA damage are presented in Table (10) as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. To describe the comet, two SCGE parameters, including tail moment (TM) and olive tail moment (OTM) were analyzed.

Tail moment (TM)

The mean value of DNA damage in terms of TM in control rats was 0.79±0.65. The mean value of comet TM measured in rats treated with iron sulfate was 25.38±4.30 which was significantly higher (P<0.001) in comparison to control groups. Interestingly, the TM induced by iron sulfate 200 mg Fe/kg was reduced by TQ post treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg which were 1.41±0.52, 30.48±11.34, 26.76±9.12, 20.36±4.56, 16.64±4.45, 12.23±5.58, 7.19±2.43, 10.89±3.49 and 16.23±4.13 respectively, and the most significant decrease in DNA damage in terms of decreased TM was 7.19±2.43 at 18 mg/kg dose of TQ in comparison with control (Table 10 and Figure 19a).

Olive tail moment (OTM)

The mean value of DNA damage in terms of OTM in control rats was 0.90±0.87. The comet OTM measured in rats treated with iron sulfate was with a mean value 29.38±4.12 which was significantly higher (P<0.001) in comparison to control groups. The OTM induced by iron sulfate 200 mg Fe/kg was reduced by TQ post treatment at the doses of 6, 9, 12, 15, 18, 21 and 24 mg/kg which were 1.99±1.01, 36.74±12.31, 29.98±10.67, 24.55±7.85, 21.62±5.69, 13.94±3.23, 9.21±1.98, 12.99±2.59 and 18.27±4.27 respectively and significant decrease in DNA damage in terms of decreased OTM was observed 9.21±1.98 at 18 mg/kg dose of TQ in comparison to iron sulfate treated groups (Table 10 and Figure 19b).

Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a dose of 200 mg/kg compared with the negative control (P<0.001). Statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the control and cells treated with
iron sulfate and TQ in the post treatment (except 21 and 24 mg/kg dose of TQ $P < 0.001$). In the presence of TQ doses (6, 9, 12, 15 and 18 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, $P < 0.001$. The protective influence of TQ on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in 18 mg/kg (Figure 19 a,b and 20).

4.2.3.6. Optimum level of TQ

The broken-line regression analysis of the post treatment with TQ levels against DNA damage induced by FeSO$_4$ data exhibited that the reduction in this parameter was best attained at 18 mg/kg of TQ. The equations and optimum level of TQ are as under,

Y=$35.31933-4.67886$, $X \leq 18$ mg/kg ($r^2=0.9979$); $Y=-20.20333+4.52$, $X \geq 18$ mg/kg ($r^2=0.99456$) $X_{opt} = 18$ mg/kg TQ (Post treatment with TQ) (TM)

Y=$41.54333-5.39143$, $X \leq 18$ mg/kg ($r^2=0.99537$); $Y=-18.22+4.53$, $X \geq 18$ mg/kg ($r^2=0.99546$) $X_{opt} = 18$ mg/kg TQ (Post treatment with TQ) (OTM)

The equations for TQ doses to DNA damage induced by FeSO$_4$ employed to calculate the optimum level of TQ, at which it shows its maximum protective effect is given in the respective figures (Figure 19 a, b).
Table 10. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone and post treatment in combination with different doses of TQ for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells/n</th>
<th>Post treatment with TQ</th>
<th>Dose (mg/kg body weight)</th>
<th>Tail moment (TM) (X±SD)</th>
<th>Olive tail moment (OTM) (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>300/6</td>
<td>---</td>
<td></td>
<td>1.41±0.52</td>
<td>1.99±1.01</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>200 mg Fe/kg</td>
<td>30.48±11.34**</td>
<td>36.74±12.31**</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₁</td>
<td>300/6</td>
<td>6 mg/kg</td>
<td>26.76±9.12**(a)</td>
<td>29.98±10.67**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₂</td>
<td>300/6</td>
<td>9 mg/kg</td>
<td>20.36±4.56**(a)</td>
<td>24.55±7.85**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₃</td>
<td>300/6</td>
<td>12 mg/kg</td>
<td>16.64±4.45**(a)</td>
<td>21.62±5.65**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₄</td>
<td>300/6</td>
<td>15 mg/kg</td>
<td>12.23±5.58**(a)</td>
<td>13.94±3.23**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₅</td>
<td>300/6</td>
<td>18 mg/kg</td>
<td>7.19±2.43**(b)</td>
<td>9.21±1.98**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₆</td>
<td>300/6</td>
<td>21 mg/kg</td>
<td>10.89±3.49**(a)</td>
<td>12.99±2.38**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄+TQ₇</td>
<td>300/6</td>
<td>24 mg/kg</td>
<td>16.23±4.13**(a)</td>
<td>18.27±4.27**(a)</td>
<td></td>
</tr>
</tbody>
</table>

Measures were calculated for 50 cells per rats (six rats per group). Values are expressed as mean ± SD and were analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. Statistically significant different compared to control: * P<0.05; ** P<0.001 and statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.001. FeSO₄=iron sulfate, TQ=thymoquinone, X=mean, SD=standard deviation.
Figure 19 a: Broken-line relationship of post treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of TQ in whole blood cells of Wistar rats assessed through comet parameter: tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001, Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.

Figure 19 b. Broken-line relationship of post treatment of TQ levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of TQ in whole blood cells of Wistar rats assessed through comet parameter: Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001, Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test TQ= thymoquinone; FeSO₄= iron sulfate; SD= standard deviation.
Figure 20. SCGE (Comet) pictures illustrating the effects of iron sulfate and thymoquinone (TQ) post treatment on the extent of DNA damage. [A] control (DMSO); [B] treated with FeSO₄ at the dose of 200 mg Fe/kg; [C, D, E, F, G, H, I] treated with TQ at the dose of 6, 9, 12, 15, 18, 21 and 24 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
PROTECTIVE EFFECTS OF QCT

4.3.1. Protective effects of QCT on chromosome aberrations induced by iron sulfate

4.3.1.1. Pre-Treatment with QCT

The types of chromosome aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by iron sulfate (200 mg/kg) alone as well as pre-treatment with the different doses of QCT are presented in Table 11. The mean number of aberrant cells was found to be 1.5 ±1.22, 112.5±33.97, 104±11.52, 99.33±17.69, 90.16±22.42, 84.16±20.74, 89.0±19.42, 97.33±14.05 and 102.8±25.02 for negative control, treatment with 200 mgFe/kg iron sulfate alone and treatment with iron sulfate (200 mgFe/kg) along with QCT pre-treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg respectively (Table 11, Figure 21 and 24). Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the total number of 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 chromosome aberrations between the control and the animals treated with iron sulfate and QCT pre-treatment (P < 0.001). In the animals treated with QCT (125, 250, 375, 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 QCT on iron sulfate induced chromosome aberrations was most prominent at the dose of QCT 500 mg/kg.

4.3.1.2. Optimum level of QCT

The broken-line regression analysis of the QCT levels against CAs induced by FeSO₄ exhibited reduction in chromosomal aberrations and maximum reduction was observed at 500 mg/kg of QCT during pre-treatment against chromosomal aberrations induced by iron sulfate. The equations and optimum level of QCT are as under,

\[ Y = 119.186 - 7.052, X \leq 500 \text{ mg/kg} \quad (r^2 = -0.99628); \quad Y = 55.56 + 6.425, X \geq 500 \text{ mg/kg} \]

\[ (r^2 = 0.99491) \quad X_{\text{opt}} = 500 \text{ mg/kg QCT (Pre-treatment with QCT)} \]

The equations for QCT doses to CAs induced by FeSO₄ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect and is given in the respective figure (Figure 21).
Table 11. The apportionment of structural aberrations in rat bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of QCT pre-treatment.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells (mg/kg body weight) Studied/n</th>
<th>Dose (mg/kg)</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>--</td>
<td>0.66±0.51</td>
<td>0.50±0.54</td>
<td>0.33±0.51</td>
<td>0.00±0.00</td>
<td>0.50±0.54</td>
<td>1.5±1.22</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>200</td>
<td>45.16±22.73</td>
<td>31.0±10.0</td>
<td>14.83±10.57</td>
<td>5.66±3.20</td>
<td>15.83±6.76</td>
<td>112.5±33.97**</td>
</tr>
<tr>
<td>FeSO₄ + QCT₁</td>
<td>600/6</td>
<td>125</td>
<td>43.16±14.86</td>
<td>29.16±8.42</td>
<td>13.33±6.40</td>
<td>5.16±2.63</td>
<td>13.16±5.84</td>
<td>104±11.52**(a)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₂</td>
<td>600/6</td>
<td>250</td>
<td>41.33±7.39</td>
<td>28.16±7.05</td>
<td>12.66±5.12</td>
<td>4.83±2.31</td>
<td>12.33±6.28</td>
<td>99.33±17.69**(b)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₃</td>
<td>600/6</td>
<td>375</td>
<td>34.33±10.09</td>
<td>26.16±10.57</td>
<td>14.33±4.88</td>
<td>4.83±3.54</td>
<td>10.50±7.34</td>
<td>90.16±22.42**(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₄</td>
<td>600/6</td>
<td>500</td>
<td>31.83±12.73</td>
<td>24.33±7.84</td>
<td>13.16±8.37</td>
<td>3.83±3.18</td>
<td>8.33±4.71</td>
<td>84.16±20.74**(e)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₅</td>
<td>600/6</td>
<td>625</td>
<td>34.66±15.70</td>
<td>24.82±5.38</td>
<td>14.50±5.46</td>
<td>4.16±1.94</td>
<td>10.83±5.43</td>
<td>89.04±19.42**(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₆</td>
<td>600/6</td>
<td>750</td>
<td>38.33±12.95</td>
<td>27.16±12.71</td>
<td>14.33±5.42</td>
<td>4.66±2.16</td>
<td>12.83±5.30</td>
<td>97.33±14.05**(b)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₇</td>
<td>600/6</td>
<td>875</td>
<td>41.00±9.89</td>
<td>28.50±9.71</td>
<td>15.16±5.24</td>
<td>5.04±2.89</td>
<td>13.16±4.07</td>
<td>102.8±25.02**(b)</td>
</tr>
</tbody>
</table>

About 600 cells were analysed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. FeSO₄=iron sulfate, QCT=quercetin, X=mean, SD=standard deviation. Statistically significant different compared to control: * P<0.05; ** P<0.001. Statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.005; (c) P<0.001.
Figure 21. Broken-line relationship of pre-treatment of QCT levels with FeSO₄ to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

Male Wistar rats received FeSO₄ alone at the dose of 200 mg Fe/kg and received pre-treatment of QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg with FeSO₄ at 200 mg Fe/kg. Data are expressed as the mean ±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. The level of significance was set at P<0.05. Broken-line regression analysis was employed to determine the optimum level of QCT. Statistically significant different compared to control: * P<0.05; ** P<0.001. Statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.005; (c) P<0.001. QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation

4.3.1.3. Simultaneous Treatment with QCT

The types of chromosome aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by iron sulfate (200 mg/kg) alone as well as simultaneous treatment with the different doses of QCT are presented in Table 12. The mean number of aberrant cells was found to be 1.66±1.21, 107.5±21.71, 95.66±24.91, 73.50±11.84, 65.0±8.41, 44.33±12.69, 59.16±12.35, 66.83±12.0 and 80.33±15.80 for negative control, treatment with 200 mgFe/kg iron sulfate alone and treatment with iron sulfate (200 mgFe/kg) along with QCT simultaneous treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg respectively (Table 12, Figure 22 and 24). Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the total number of 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 chromosome aberrations between the control and the animals treated...
with iron sulfate and QCT simultaneous treatment ($P < 0.001$). In the animals treated with QCT (125, 250, 375, 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 greatest reduction of chromosome aberrations was obtained in the QCT 500 mg/kg. The ameliorating effect of QCT on iron sulfate induced chromosome aberrations was most prominent in simultaneous treatment.

4.3.1.4. Optimum level of QCT

The broken-line regression analysis of the QCT levels against chromosomal aberrations induced by FeSO$_4$ exhibited reduction in chromosomal aberrations and maximum reduction was observed at 500 mg/kg of QCT during simultaneous treatment against chromosomal aberrations induced by iron sulfate. The equations and optimum level of QCT are as under,

\[ Y = 124.298 - 15.7, \quad X \leq 500 \text{ mg/kg} \quad (r^2 = 0.99259); \quad Y = -12.523 + 11.567, \quad X \geq 500 \text{ mg/kg} \quad (r^2 = 0.99343) \]

$X_{opt} = 500 \text{ mg/kg QCT (Simultaneous treatment with QCT)}$

The equations for QCT doses to chromosomal aberrations induced by FeSO$_4$ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect and is given in the respective figure (Figure 22).
Table 12. The apportionment of structural aberrations in rat bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of QCT simultaneous treatment.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells (mg/kg body weight)</th>
<th>Dose</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>---</td>
<td>0.50±0.54</td>
<td>0.33±0.51</td>
<td>0.59±0.54</td>
<td>0.00±0.00</td>
<td>0.33±0.51</td>
<td>1.66±1.21</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6</td>
<td>200 mg Fe/kg</td>
<td>45.0±12.75</td>
<td>31.0±8.09</td>
<td>12.50±5.04</td>
<td>5.16±2.48</td>
<td>13.83±5.11</td>
<td>107.5±21.71**</td>
</tr>
<tr>
<td>FeSO₄+QCT₁</td>
<td>600/6</td>
<td>125 mg/kg</td>
<td>40.33±5.25</td>
<td>28.50±6.71</td>
<td>10.50±3.98</td>
<td>4.83±1.94</td>
<td>11.50±4.39</td>
<td>95.66±24.91** (a)</td>
</tr>
<tr>
<td>FeSO₄+QCT₂</td>
<td>600/6</td>
<td>250 mg/kg</td>
<td>29.50±5.61</td>
<td>20.0±8.85</td>
<td>9.50±3.08</td>
<td>4.33±1.86</td>
<td>10.16±4.25</td>
<td>73.50±11.84** (a)</td>
</tr>
<tr>
<td>FeSO₄+QCT₃</td>
<td>600/6</td>
<td>375 mg/kg</td>
<td>25.50±3.72</td>
<td>18.16±7.41</td>
<td>8.0±3.34</td>
<td>4.0±1.54</td>
<td>9.33±3.82</td>
<td>65.0±8.41** (b)</td>
</tr>
<tr>
<td>FeSO₄+QCT₄</td>
<td>600/6</td>
<td>500 mg/kg</td>
<td>14.83±4.95</td>
<td>15.0±4.19</td>
<td>5.50±1.87</td>
<td>2.50±1.51</td>
<td>6.50±3.08</td>
<td>44.33±12.69** (b)</td>
</tr>
<tr>
<td>FeSO₄+QCT₅</td>
<td>600/6</td>
<td>625 mg/kg</td>
<td>21.66±3.77</td>
<td>18.16±3.81</td>
<td>8.33±3.14</td>
<td>3.83±1.94</td>
<td>7.16±2.78</td>
<td>59.16±12.35** (b)</td>
</tr>
<tr>
<td>FeSO₄+QCT₆</td>
<td>600/6</td>
<td>750 mg/kg</td>
<td>24.33±5.88</td>
<td>19.0±7.92</td>
<td>10.16±4.44</td>
<td>4.50±2.34</td>
<td>8.83±3.60</td>
<td>66.83±12.0* (b)</td>
</tr>
<tr>
<td>FeSO₄+QCT₇</td>
<td>600/6</td>
<td>875 mg/kg</td>
<td>29.66±10.32</td>
<td>22.66±6.28</td>
<td>11.16±3.48</td>
<td>5.04±1.41</td>
<td>11.83±4.48</td>
<td>80.33±15.80** (a)</td>
</tr>
</tbody>
</table>

About 600 cells were analysed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. FeSO₄=iron sulfate, QCT=quecetin, X=mean, SD=standard deviation.
Statistically significant different compared to control: * P<0.05; ** P<0.001
Statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.001.
Figure 22. Broken-line relationship of simultaneous treatment of QCT levels with FeSO₄ to reduction in chromosomal aberrations in bone marrow cells of Wistar rats.

Male Wistar rats received FeSO₄ alone at the dose of 200 mg Fe/kg and received simultaneous treatment of QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg with FeSO₄ at 200 mg Fe/kg. Data are expressed as the mean ±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. The level of significance was set at P<0.05. Broken-line regression analysis was employed to determine the optimum level of QCT. Statistically significant different compared to control: * P<0.05; ** P<0.001. Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.001. QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation

4.3.1.5. Post Treatment with QCT

The types of chromosome aberrations, with special emphasis on breaks, exchanges forming dicentrics, fragments and sister chromatid union forming rings induced by iron sulfate (200 mg/kg) alone as well as post treatment with the different doses of QCT are presented in Table 13. The mean number of aberrant cells was found to be 2.0 ±0.89, 107.6±15.20, 99.83±15.94, 90.0±13.16, 78.50±10.36, 68.0±7.01, 76.50±6.22, 85.16±5.41 and 98.83±8.47 for negative control, treatment with 200 mgFe/kg iron sulfate alone and treatment with iron sulfate (200 mgFe/kg) along with QCT post treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg respectively (Table 13, Figure 23 and 24). Iron sulfate at a concentration of 200 mg/kg produced a highly statistically significant increase in the total number of 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 chromosome aberrations between the control and the animals treated with
iron sulfate and QCT post treatment ($P < 0.001$). In the animals treated with QCT (125, 250, 375, 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 QCT on iron sulfate induced chromosome aberrations was most prominent at the dose of QCT 500 mg/kg.

4.3.1.6. Optimum level of QCT

The broken-line regression analysis of the QCT levels against chromosomal aberrations induced by FeSO$_4$ exhibited reduction in chromosomal aberrations and maximum reduction was observed at 500 mg/kg of QCT during post treatment against chromosomal aberrations induced by iron sulfate. The equations and optimum level of QCT are as under,

\[ Y = 118.945 - 10.053, \quad X \leq 500 \text{ mg/kg} \ (r^2 = 0.99771); \quad Y = 16.375 + 10.115, \quad X \geq 500 \text{ mg/kg} \]

($r^2 = 0.99241$) $X_{opt} = 500 \text{ mg/kg QCT (Post treatment with QCT)}$

The equations for QCT doses to chromosomal aberrations induced by FeSO$_4$ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect and is given in the respective figure (Figure 23).
Table 13. The apportionment of structural aberrations in rat bone marrow cells of Wistar rats after 24 h of iron sulfate treatment along with different doses of QCT post treatment.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h dose (mg/kg body weight)</th>
<th>Total cells studied/n</th>
<th>Chromatid breaks (X±SD)</th>
<th>Chromosome breaks (X±SD)</th>
<th>Acentric fragments (X±SD)</th>
<th>Dicentric chromosomes (X±SD)</th>
<th>Chromosome rings (X±SD)</th>
<th>Total aberrations (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>600/6</td>
<td>0.66±0.51</td>
<td>0.50±0.83</td>
<td>0.33±0.51</td>
<td>0.16±0.40</td>
<td>0.33±0.51</td>
<td>0.33±0.51</td>
<td>2.0±0.89</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>600/6 200 mg Fe/kg</td>
<td>45.33±6.15</td>
<td>33.16±7.13</td>
<td>14.0±3.74</td>
<td>5.50±1.87</td>
<td>9.66±2.73</td>
<td>2.17±1.08</td>
<td>107.6±15.20**</td>
</tr>
<tr>
<td>FeSO₄ + QCT₁</td>
<td>600/6 125 mg/kg</td>
<td>43.50±3.50</td>
<td>30.50±4.96</td>
<td>13.16±2.48</td>
<td>4.66±2.50</td>
<td>8.0±1.41</td>
<td>89.8±11.26** (a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₂</td>
<td>600/6 250 mg/kg</td>
<td>40.16±7.30</td>
<td>27.83±4.35</td>
<td>11.33±2.16</td>
<td>3.50±0.04</td>
<td>7.16±1.47</td>
<td>80.0±13.16** (a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₃</td>
<td>600/6 375 mg/kg</td>
<td>36.33±4.17</td>
<td>25.16±3.43</td>
<td>8.66±2.16</td>
<td>3.16±0.75</td>
<td>5.16±0.75</td>
<td>78.5±10.36** (a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₄</td>
<td>600/6 500 mg/kg</td>
<td>31.53±2.42</td>
<td>22.50±4.18</td>
<td>7.66±1.21</td>
<td>2.50±0.83</td>
<td>4.0±0.63</td>
<td>68.0±7.01** (b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₅</td>
<td>600/6 625 mg/kg</td>
<td>34.83±2.31</td>
<td>25.16±1.22</td>
<td>8.16±0.98</td>
<td>3.66±0.81</td>
<td>4.66±0.81</td>
<td>75.5±16.62** (b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₆</td>
<td>600/6 750 mg/kg</td>
<td>38.33±2.16</td>
<td>27.83±2.99</td>
<td>9.16±2.31</td>
<td>4.16±0.75</td>
<td>5.66±0.81</td>
<td>85.1±65.41** (a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₇</td>
<td>600/6 875 mg/kg</td>
<td>43.16±3.92</td>
<td>29.66±3.38</td>
<td>12.83±1.94</td>
<td>4.83±1.16</td>
<td>8.33±1.21</td>
<td>98.8±8.47** (a)</td>
<td></td>
</tr>
</tbody>
</table>

About 600 cells were analysed per treatment for the total number of structural aberrations. Data are expressed as the X±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Turkey post hoc test was used to examine the differences between samples. FeSO₄= iron sulfate, QCT= quercetin, X=mean, SD= standard deviation. Statistically significant different compared to control: * P<0.05; ** P<0.001 Statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.001.
Figure 23. Broken-line relationship of post treatment of QCT levels with FeSO₄ to reduction in chromosomal aberrations in bone marrow cells of Wist rat.

Male Wistar rats received FeSO₄ alone at the dose of 200 mg Fe/kg and received post treatment of QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg with FeSO₄ at 200 mg Fe/kg. Data are expressed as the mean±SD and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. The level of significance was set at \( P<0.05 \). Broken-line regression analysis was employed to determine the optimum level of QCT. Statistically significant different compared to control: * \( P<0.05 \); ** \( P<0.001 \). Statistically significant different from FeSO₄ groups: (a) \( P<0.05 \); (b) \( P<0.001 \). QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Figure 24. Photomicrographs illustrating the effects of iron sulfate (FeSO₄) and quercetin (QCT) on bone marrow cells of Wistar rats (A) Acentric fragment; (B) Chromosome break; Ring chromosome (C) Acentric fragment (D) Dicentric chromosomes.
4.3.2. Protective effects of QCT on micronucleus induced by iron sulfate

4.3.2.1. Pre-Treatment with QCT

The number of MNPCEs among 2000 PCE, indicative of genotoxicity, is presented in Table 14. The mean value of MNPCEs was found to be 2.0 ± 1.6, 30.5 ± 3.1, 27.0 ± 2.9, 21.5 ± 3.3, 17.6 ± 1.8, 11.66 ± 1.4, 14.83 ± 2.6, 19.16 ± 2.2 and 22.33 ± 3.5 for negative control, 200 mgFe/kg iron sulfate alone, cells treated with iron sulfate (200 mgFe/kg) and pre-treatment with 125, 250, 375, 500, 625, 750 and 875 mg/kg doses of QCT respectively (Table 14, Figure 25 and 28). Iron sulfate induced a significant (P<0.001) increase in MN-PCE number at a dose of 200 mg/kg. QCT at the doses of 125, 250, 375, and 500 mg/kg, induced statistically significant decreases in the mean value of MN induced by iron sulfate in pre-treatments. QCT showed significant inhibitory effect on iron sulfate induced MN in PCE. All doses of QCT tested were found to be effective in reducing the mean value of MN induced by iron sulfate except 625, 750 and 875 mg/kg doses. With iron sulfate the lower doses of QCT were not effective in reducing the mean value of MN. The most significant inhibitory effect on MN formation in PCE (P<0.001) was observed at 500 mg/kg dose of QCT. The protective influence of QCT on iron sulfate induced in the mean value of MNPCEs was most prominent at 500 mg/kg (Figure 25).

4.3.2.2. Optimum level of QCT

The broken-line regression analysis of the QCT levels against MNPCEs induced by FeSO₄ exhibited reduction in this parameter was best attained at 500 mg/kg of QCT during pre-treatment against genotoxicity induced by iron sulfate. The equations and optimum level of QCT are as under,

\[ Y = 35.76667 - 4.7, \ X \leq 500 \ \text{mg/kg} \ (r^2 = 0.9964); \ Y = -6.61667 + 3.63333, \ X \geq 500 \ \text{mg/kg} \ (r^2 = 0.99794) \]

\[ X_{opt} = 500 \ \text{mg/kg QCT (Pre-treatment with QCT)} \]

The equations for QCT doses to MNPCEs induced by FeSO₄ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect is given in the respective figure (Figure 25).
4.3.2.3. Simultaneous Treatment with QCT

The number of MNPCEs among 2000 PCE, indicative of genotoxicity, is presented in Table 14. The mean value of MNPCEs was found to be 1.5 ± 0.8, 29.16 ± 3.9, 22.0 ± 3.2, 18.5 ± 2.9, 14.5 ± 2.2, 9.5 ± 2.4, 12.8 ± 3.1, 17.16 ± 2.6 and 20.5 ± 3.3 for negative control, 200 mgFe/kg iron sulfate alone, cells treated with iron sulfate (200 mgFe/kg) and simultaneous treatment with 125, 250, 375, 500, 625, 750 and 875 mg/kg doses of QCT respectively (Table 14, Figure 26 and 28). Iron sulfate induced a significant (P<0.001) increase in MNPCEs number at a dose of 200 mg/kg. QCT at the doses of 125, 250, 375, and 500 mg/kg, induced statistically significant decreases in the mean value of MNPCEs induced by iron sulfate in simultaneous treatment. QCT showed significant inhibitory effect on iron sulfate induced MN in PCE. All doses of QCT tested were found to be effective in reducing the mean value of MNPCEs induced by iron sulfate except 625, 750 and 875 mg/kg doses. With iron sulfate the lower doses of QCT were not effective in reducing the mean value of MNPCEs. The most significant inhibitory effect on MN formation in PCE (P<0.001) was observed at 500 mg/kg dose of QCT. The protective influence of QCT on iron sulfate induced MN formation was most prominent in the simultaneous treatments (Figure 26).

4.3.2.4. Optimum level of QCT

The broken-line regression analysis of the QCT levels against MNPCEs induced by FeSO₄ exhibited reduction in this parameter was best attained at 500 mg/kg of QCT during simultaneous treatment against genotoxicity induced by iron sulfate. The equations and optimum level of QCT are as under,

\[ Y = 32.78333 - 4.68333, \quad X \leq 500 \text{ mg/kg} \quad (r^2 = 0.99229); \quad Y = -9.26667 + 3.73333, \quad X \geq 500 \text{ mg/kg} \]

\[ (r^2 = 0.99857) \text{ } X_{\text{opt}} = 500 \text{ mg/kg QCT (Simultaneous treatment with QCT)} \]

The equations for QCT doses to MNPCEs induced by FeSO₄ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect is given in the respective figure (Figure 26).
4.3.2.5. Post Treatment with QCT

The number of MNPCES among 2000 PCE, indicative of genotoxicity, is presented in Table 14. The mean value of MNPCES was found to be 1.66 ± 0.9, 31.0 ± 2.9, 28.16 ± 2.2, 22.16 ± 1.9, 18.16 ± 2.8, 12.66 ± 1.4, 16.83 ± 2.6, 19.0 ± 2.0 and 23.16 ±3.6 for negative control, 200 mgFe/kg iron sulfate alone, cells treated with iron sulfate (200 mgFe/kg) and post treatment with 125, 250, 375, 500, 625, 750 and 875 mg/kg doses of QCT respectively (Table 14, Figure 27 and 28). Iron sulfate induced a significant (P<0.001) increase in MNPCES number at a dose of 200 mg/kg. QCT at the doses of 125, 250, 375, and 500 mg/kg, induced statistically significant decreases in the mean value of MNPCES induced by iron sulfate in post treatment. QCT showed significant inhibitory effect on iron sulfate induced MN in PCE. All doses of QCT tested were found to be effective in reducing the mean value of MNPCES induced by iron sulfate except 625, 750 and 875 mg/kg doses. With iron sulfate the lower doses of QCT were not effective in reducing the mean value of MNPCES. The most effectively significant inhibitory effect on MN in PCE (P<0.001) was observed at 500 mg/kg dose of QCT. The protective influence of QCT on iron sulfate induced in the mean value of MNPCES was most prominent at 500 mg/kg (Figure 27).

4.3.2.6. Optimum level of QCT

The broken-line regression analysis of the QCT levels against MNPCES induced by FeSO$_4$ exhibited reduction in this parameter was best attained at 500 mg/kg of QCT during post treatment against genotoxicity induced by iron sulfate. The equations and optimum level of QCT are as under,

\[Y = 36.43333-4.66667, X \leq 500 \text{ mg/kg (} r^2=0.99511); Y = -3.96667+3.36667, X \geq 500 \text{ mg/kg}\]

\[(r^2=0.99302) \ X_{\text{opt}} = 500 \text{ mg/kg QCT (Post treatment with QCT)}\]

The equations for QCT doses to MNPCES induced by FeSO$_4$ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect is given in the respective figure (Figure 27).
<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>MNPCs per 2000 Polychromatic Erythrocytes</th>
<th>Dose (mg/kg body weight)</th>
<th>At 24 h</th>
<th>Total PCE's</th>
<th>Pre-treatment with QCT</th>
<th>Post treatment with QCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>1200/0/6</td>
<td>200 mg Fe/kg</td>
<td>20 ± ± 1.6</td>
<td>30.5 ± ± 3.1***</td>
<td>21.6 ± ± 3.3***(c)</td>
<td>14.5 ± ± 3.2***(c)</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>1200/0/6</td>
<td>125 mg/kg</td>
<td>27.0 ± ± 2.9***(c)</td>
<td>21.6 ± ± 3.3***(c)</td>
<td>18.5 ± ± 2.9***(c)</td>
<td>12.6 ± ± 2.9***(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT</td>
<td>1200/0/6</td>
<td>250 mg/kg</td>
<td>11.6 ± ± 1.4***(c)</td>
<td>12.8 ± ± 3.1***(c)</td>
<td>17.1 ± ± 2.6***(c)</td>
<td>19.0 ± ± 2.0***(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT</td>
<td>1200/0/6</td>
<td>375 mg/kg</td>
<td>14.8 ± ± 2.6***(c)</td>
<td>19.1 ± ± 2.6***(c)</td>
<td>22.3 ± ± 3.5***(c)</td>
<td>20.5 ± ± 3.3***(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT</td>
<td>1200/0/6</td>
<td>500 mg/kg</td>
<td>22.3 ± ± 3.5***(c)</td>
<td>20.5 ± ± 3.3***(c)</td>
<td>17.1 ± ± 2.6***(c)</td>
<td>19.0 ± ± 2.0***(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT</td>
<td>1200/0/6</td>
<td>625 mg/kg</td>
<td>22.3 ± ± 3.5***(c)</td>
<td>20.5 ± ± 3.3***(c)</td>
<td>17.1 ± ± 2.6***(c)</td>
<td>19.0 ± ± 2.0***(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT</td>
<td>1200/0/6</td>
<td>750 mg/kg</td>
<td>22.3 ± ± 3.5***(c)</td>
<td>20.5 ± ± 3.3***(c)</td>
<td>17.1 ± ± 2.6***(c)</td>
<td>19.0 ± ± 2.0***(c)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of 6 Wistar rats in each group and were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons. Tukey post hoc test was used to examine the differences between samples. P values: *P < 0.05; **P < 0.01, and statistically significant different from FeSO₄ group. (a) P < 0.05; (b) P < 0.01; (c) P < 0.001.
Figure: Broken-Line relationship of pre, simultaneous and post treatment of QCT - mediated modification of iron sulfate induced micronuclei in polychromatic erythrocytes (PCEs) in bone marrow cells of Wistar rats.
4.3.3. Protective effect of QCT on DNA damage induced by iron sulfate

4.3.3.1. Pre Treatment with QCT

The effects of iron sulfate and QCT treatment on the extent of DNA damage are presented in Table 15 as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. To describe the comet, two SCGE parameters, including tail moment (TM) and olive tail moment (OTM) were analyzed.

Tail moment (TM)

The mean value of DNA damage in terms of TM in control rats was 0.79±0.65. The mean value of comet TM measured in rats treated with iron sulfate was 25.38±4.30 which was significantly higher (P < 0.001) in comparison to control groups. Interestingly, the TM induced by iron sulfate 200 mg Fe/kg was reduced by QCT pretreatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg which were 0.98 ± 0.9, 32.66 ± 3.4, 25.62 ± 3.1, 17.72 ± 2.8, 13.94 ± 1.9, 5.96 ± 1.1, 9.64 ± 2.4, 11.40 ± 2.1 and 16.33 ± 3.2 respectively, and the most significant decrease in DNA damage in terms of decreased TM was 5.96 ± 1.1 at 500 mg/kg dose of QCT in comparison with control (Table 15 and Figure 29a).

Olive tail moment (OTM)

The mean value of DNA damage in terms of OTM in control rats was 0.90±0.87. The comet OTM measured in rats treated with iron sulfate was with a mean value 29.38±4.12 which was significantly higher (P < 0.001) in comparison to control groups. The OTM induced by iron sulfate 200 mg Fe/kg was reduced by QCT pretreatment at the doses of 125, 250, 375, 500, 625, 750 and 875 which were 1.86 ± 1.2, 36.22± 3.7, 27.04 ± 2.8, 23.12 ± 3.1, 15.96 ± 2.3, 7.66 ± 1.9, 14.12± 2.4, 17.30 ± 3.2 and 24.9 ± 3.8 respectively and significant decrease in DNA damage in terms of decreased OTM was observed 7.66 ± 1.9 at 500 mg/kg dose of QCT in comparison to iron sulfate treated groups (Table 15 and Figure 29b).

Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a dose of 200 mg/kg compared with the negative control (P <0.001). Statistical analysis showed that there are significant differences in
the DNA damage in terms of TM and OTM between the control and cells treated with iron sulfate and QCT in the pre-treatments (except 625, 750 and 875 mg/kg dose of QCT \( P < 0.001 \)). In the presence of QCT doses (6, 9, 12, 15 and 500 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, \( P < 0.001 \). The protective influence of QCT on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in 500 mg/kg (Figure 29 a,b and 30).

4.3.3.2. Optimum level of QCT

The broken-line regression analysis of pre-treatment with QCT levels against DNA damage induced by FeSO₄ data exhibited that the reduction in this parameter was best attained at 500 mg/kg of QCT. The equations and optimum level of QCT are as under,

\[
\begin{align*}
\text{Y} &= 38.704 - 6.508, \quad X \leq 500 \text{ mg/kg} \quad (r^2 = 0.99531); \\
\text{Y} &= -10.488 + 3.283, \quad X \geq 500 \text{ mg/kg} \\
(r^2 = 0.98429) & \quad X_{opt} = 500 \text{ mg/kg QCT (TM)} \\
\text{Y} &= 42.46 - 6.82, \quad X \leq 500 \text{ mg/kg} \quad (r^2 = 0.99376); \\
\text{Y} &= -19.64 + 5.49, \quad X \geq 500 \text{ mg/kg} \\
(r^2 = 0.98926) & \quad X_{opt} = 500 \text{ mg/kg QCT (OTM)}
\end{align*}
\]

The equations for QCT doses to DNA damage induced by FeSO₄ employed to calculate the optimum level of QCT, at which it shows its maximum protective effect is given in the respective figures (Figure 29 a,b).
Table 15. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone or in combination of pre-treatment with different doses of QCT for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>Pre-treatment with QCT</th>
<th>( \text{At 24 h} )</th>
<th>Dose (mg/kg body weight)</th>
<th>Tail moment (TM) (X±SD)</th>
<th>Olive tail moment (OTM) (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>300/6</td>
<td>---</td>
<td>0.98 ± 0.9</td>
<td>1.86 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>200 mg Fe/kg</td>
<td>32.66 ± 3.4**</td>
<td>36.22 ± 3.7**</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₁</td>
<td>300/6</td>
<td>125 mg/kg</td>
<td>25.62 ± 3.1**(a)</td>
<td>27.04 ± 2.8**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₂</td>
<td>300/6</td>
<td>250 mg/kg</td>
<td>17.72 ± 2.8**(b)</td>
<td>23.12 ± 3.1**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₃</td>
<td>300/6</td>
<td>375 mg/kg</td>
<td>13.94 ± 1.9**(c)</td>
<td>15.96 ± 2.2**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₄</td>
<td>300/6</td>
<td>500 mg/kg</td>
<td>5.96 ± 1.1**(c)</td>
<td>7.66 ± 1.9**(c)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₅</td>
<td>300/6</td>
<td>625 mg/kg</td>
<td>9.64 ± 2.4**(c)</td>
<td>14.12± 2.4**(c)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₆</td>
<td>300/6</td>
<td>750 mg/kg</td>
<td>11.40 ± 2.1**(c)</td>
<td>17.30 ± 2.2**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₇</td>
<td>300/6</td>
<td>875 mg/kg</td>
<td>16.33 ± 3.2**(a)</td>
<td>24.9 ± 3.8**(a)</td>
<td></td>
</tr>
</tbody>
</table>

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameters: Tail moment and Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * \( P<0.05 \); ** \( P<0.001 \), Statistically significant different from FeSO₄ groups; (a) \( P<0.05 \); (b) \( P<0.005 \); (c) \( P<0.001 \), as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Chapter 4: Results

Figure 29 (a): Broken-line relationship of pre-treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: Tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameter: tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P < 0.05; ** P < 0.001, Statistically significant different from FeSO₄ groups: (a) P < 0.05; (b) P < 0.005; (c) P < 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.

Figure 29 (b): Broken-line relationship of pre-treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameter: Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P < 0.05; ** P < 0.001, Statistically significant different from FeSO₄ groups: (a) P < 0.05; (b) P < 0.005; (c) P < 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Figure 30. SCGE (Comet) pictures illustrating the effects of iron sulfate and quercetin (QCT) pre-treatment on the extent of DNA damage. [A] control (DMSO); [B] treated with FeSO₄ at the dose of 200 mgFe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
4.3.3.3. Simultaneous Treatment with QCT

The effects of iron sulfate and QCT treatment on the extent of DNA damage are presented in Table 16 as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. To describe the comet, two SCGE parameters, including tail moment (TM) and olive tail moment (OTM) were analyzed.

**Tail moment (TM)**

The mean value of DNA damage in terms of TM in control rats was $0.79 \pm 0.65$. The mean value of comet TM measured in rats treated with iron sulfate was $25.38 \pm 4.30$ which was significantly higher ($P < 0.001$) in comparison to control groups. Interestingly, the TM induced by iron sulfate 200 mg Fe/kg was reduced by QCT simultaneous treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg which were $1.33 \pm 0.8$, $31.57 \pm 2.8$, $26.85 \pm 3.7$, $18.28 \pm 2.2$, $12.21 \pm 2.1$, $4.94 \pm 0.9$, $7.82 \pm 1.3$, $8.99 \pm 2.4$ and $11.93 \pm 2.9$ respectively, and the most significant decrease in DNA damage in terms of decreased TM was $4.94 \pm 0.9$ at 500 mg/kg dose of QCT in comparison with control (Table 16 and Figure 31a).

**Olive tail moment (OTM)**

The mean value of DNA damage in terms of OTM in control rats was $0.90 \pm 0.87$. The comet OTM measured in rats treated with iron sulfate was with a mean value $29.38 \pm 4.12$ which was significantly higher ($P < 0.001$) in comparison to control groups. The OTM induced by iron sulfate 200 mg Fe/kg was reduced by QCT simultaneous treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg which were $1.77 \pm 0.7$, $37.74 \pm 3.3$, $30.18 \pm 3.1$, $24.84 \pm 2.9$, $15.71 \pm 2.4$, $6.98 \pm 2.1$, $10.91 \pm 2.0$, $12.59 \pm 2.2$ and $14.60 \pm 4.0$ respectively and significant decrease in DNA damage in terms of decreased OTM was observed $6.98 \pm 2.1$ at 500 mg/kg dose of QCT in comparison to iron sulfate treated groups (Table 16 and Figure 31b).

Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a dose of 200 mg/kg compared with the negative control ($P < 0.001$). Statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the control and cells treated with
iron sulfate and QCT in the simultaneous treatment (except 625, 750 and 875 mg/kg
dose of QCT ($P < 0.001$). In the presence of QCT doses (125, 250, 375, and 500
mg/kg), the DNA damage in terms of TM and OTM statistically significantly
decreased when compared with the animal treated with iron sulfate at a dose of 200
mg/kg alone, $P < 0.001$. The protective influence of QCT on iron sulfate induced
DNA damage in terms of TM and OTM was most prominent in simultaneous
treatment (Figure 31 a, b and 32).

4.3.3.4. Optimum level of QCT

The broken-line regression analysis of the simultaneous treatment with QCT levels
against DNA damage induced by FeSO$_4$ data exhibited that the reduction in this
parameter was best attained at 500 mg/kg of QCT. The equations and optimum level
of QCT are as under,

$$Y = 39.14-6.79, X \leq 500 \text{ mg/kg ($r^2=0.99716$); } Y = -5.971+2.214, X \geq 500 \text{ mg/kg}$$

($r^2=0.98785$) $X_{opt} = 500 \text{ mg/kg QCT (TM)}$

$$Y = 45.887-7.599, X \leq 500 \text{ mg/kg ($r^2=0.99612$); } Y = -4.681+2.454, X \geq 500 \text{ mg/kg}$$

($r^2=0.9798$) $X_{opt} = 500 \text{ mg/kg QCT (OTM)}$

The equations for QCT doses to DNA damage induced by FeSO$_4$ employed to
calculate the optimum level of QCT, at which it shows its maximum protective effect
is given in the respective figures (Figure 31 a, b).
Table 16. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone or in combination of simultaneous treatment with different doses of QCT for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>Simultaneous treatment with QCT</th>
<th>At 24 h</th>
<th>Dose (mg/kg body weight)</th>
<th>Tail moment (TM) (X±SD)</th>
<th>Olive tail moment (OTM) (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>30/06</td>
<td>---</td>
<td>1.33 ± 0.8</td>
<td>1.77 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
<td>30/06</td>
<td>200 mg Fe/kg</td>
<td>31.57 ± 2.8**</td>
<td>37.74± 3.3**</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₁</td>
<td>30/06</td>
<td>125 mg/kg</td>
<td>26.85 ± 3.7**(a)</td>
<td>30.18 ± 3.1**(a)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₂</td>
<td>30/06</td>
<td>250 mg/kg</td>
<td>18.28 ± 2.2**(b)</td>
<td>24.84 ± 2.5**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₃</td>
<td>30/06</td>
<td>375 mg/kg</td>
<td>12.21 ± 2.1**(c)</td>
<td>15.71 ± 2.4**(b)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₄</td>
<td>30/06</td>
<td>500 mg/kg</td>
<td>4.94 ± 0.9*(c)</td>
<td>6.98 ± 2.1*(c)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₅</td>
<td>30/06</td>
<td>625 mg/kg</td>
<td>7.82 ± 1.3*(c)</td>
<td>10.91± 2.0**(c)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₆</td>
<td>30/06</td>
<td>750 mg/kg</td>
<td>8.99 ± 2.4**(c)</td>
<td>12.59 ± 2.2**(c)</td>
<td></td>
</tr>
<tr>
<td>FeSO₄ + QCT₇</td>
<td>30/06</td>
<td>875 mg/kg</td>
<td>11.93 ± 2.9**(c)</td>
<td>14.60 ± 4.0***(c)</td>
<td></td>
</tr>
</tbody>
</table>

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameters: Tail moment and Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P<0.05; ** P<0.001, Statistically significant different from FeSO₄ groups: (a) P<0.05; (b) P<0.005; (c) P<0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Figure 31 (a): Broken-line relationship of simultaneous treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: tail moment.

DNA damage induced by and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameter: tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P < 0.05; ** P < 0.001, Statistically significant different from FeSO₄ groups: (a) P < 0.05; (b) P < 0.005; (c) P < 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.

Figure 31 (b): Broken-line relationship of simultaneous treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: olive tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameter: Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P < 0.05; ** P < 0.001, Statistically significant different from FeSO₄ groups: (a) P < 0.05; (b) P < 0.005; (c) P < 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Figure 32. SCGE (Comet) pictures illustrating the effects of iron sulfate and quercetin (QCT) simultaneous treatment on the extent of DNA damage. [A] control (DMSO); [B] treated with FeSO₄ at the dose of 200 mg Fe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
4.3.3.5. Post Treatment with QCT

The effects of iron sulfate and QCT treatment on the extent of DNA damage are presented in Table (17) as mean values of tail moment and olive tail moment for each rat. The data were analyzed by using CASP software. To describe the comet, two SCGE parameters, including tail moment (TM) and olive tail moment (OTM) were analyzed.

Tail moment (TM)

The mean value of DNA damage in terms of TM in control rats was 0.79±0.65. The mean value of comet TM measured in rats treated with iron sulfate was 25.38±4.30 which was significantly higher (P < 0.001) in comparison to control groups. Interestingly, the TM induced by iron sulfate 200 mg Fe/kg was reduced by QCT post treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg which were 1.24 ± 0.4, 33.24 ± 4.1, 24.20 ± 3.7, 18.45 ± 2.3, 12.63 ± 3.1, 6.56 ± 2.1, 10.21 ± 1.6, 15.74 ± 2.3 and 17.64 ± 3.3 respectively, and the most significant decrease in DNA damage in terms of decreased TM was 6.56 ± 2.1 at 500 mg/kg dose of QCT in comparison with control (Table 17 and Figure 33a).

Olive tail moment (OTM)

The mean value of DNA damage in terms of OTM in control rats was 0.90±0.87. The comet OTM measured in rats treated with iron sulfate was with a mean value 29.38±4.12 which was significantly higher (P < 0.001) in comparison to control groups. The OTM induced by iron sulfate 200 mg Fe/kg was reduced by QCT post treatment at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg which were 1.79 ± 1.1, 38.67± 3.1, 28.78 ± 3.5, 23.79 ± 2.9, 16.34 ± 3.7, 8.83 ± 2.2, 13.68± 2.5, 19.45 ± 3.0 and 23.14 ± 3.1 respectively and significant decrease in DNA damage in terms of decreased OTM was observed 8.83 ± 2.2 at 500 mg/kg dose of QCT in comparison to iron sulfate treated groups (Table 17 and Figure 33 b).

Significantly higher levels of DNA damage in terms of TM and OTM were detected in rats exposed to iron sulfate at a dose of 200 mg/kg compared with the negative control (P <0.001). Statistical analysis showed that there are significant differences in the DNA damage in terms of TM and OTM between the control and cells treated with
iron sulfate and QCT in the post treatment (except 625, 750 and 875 mg/kg dose of QCT \( P < 0.001 \)). In the presence of QCT doses (125, 250, 375, and 500 mg/kg), the DNA damage in terms of TM and OTM statistically significantly decreased when compared with the animal treated with iron sulfate at a dose of 200 mg/kg alone, \( P < 0.001 \). The protective influence of QCT on iron sulfate induced DNA damage in terms of TM and OTM was most prominent in 500 mg/kg (Figure 33 a,b and 34).

4.3.3.6. Optimum level of QCT

The broken-line regression analysis of the post treatment with QCT levels against DNA damage induced by FeSO\(_4\) data exhibited that the reduction in this parameter was best attained at 500 mg/kg of QCT. The equations and optimum level of QCT are as under,

\[
Y = 38.495 - 6.493, X \leq 500 \text{ mg/kg} \quad (r^2=0.99545) \quad Y = -12.663 + 3.877, X \geq 500 \text{ mg/kg} \\
(r^2=0.98515) \quad X_{\text{opt}} = 500 \text{ mg/kg} \quad \text{QCT (TM)}
\]

\[
Y = 44.918 - 7.212, X \leq 500 \text{ mg/kg} \quad (r^2=0.99599) \quad Y = -15.38 + 4.87, X \geq 500 \text{ mg/kg} \\
(r^2=0.9967) \quad X_{\text{opt}} = 500 \text{ mg/kg} \quad \text{QCT (OTM)}
\]

The equations for QCT doses to DNA damage induced by FeSO\(_4\) employed to calculate the optimum level of QCT, at which it shows its maximum protective effect is given in the respective figures (Figure 33 a, b).
Table 17. Mean and standard deviation of comet tail moment and olive tail moment of cells from the whole blood of Wistar rats induced by iron sulfate alone or in combination of post treatment with different doses of QCT for 24 h.

<table>
<thead>
<tr>
<th>Treated Groups</th>
<th>At 24 h Total cells/n</th>
<th>Dose (mg/kg body weight)</th>
<th>Tail moment (TM) (X±SD)</th>
<th>Olive tail moment (OTM) (X±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>300/6</td>
<td>---</td>
<td>1.24 ± 0.4</td>
<td>1.79 ± 1.1</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>300/6</td>
<td>200 mg Fe/kg</td>
<td>33.24 ± 4.1**</td>
<td>38.67 ± 3.1**</td>
</tr>
<tr>
<td>FeSO₄ + QCT₁</td>
<td>300/6</td>
<td>125 mg/kg</td>
<td>24.20 ± 3.7**(a)</td>
<td>28.78 ± 3.5**(a)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₂</td>
<td>300/6</td>
<td>250 mg/kg</td>
<td>18.45 ± 2.3**(b)</td>
<td>23.79 ± 2.9**(b)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₃</td>
<td>300/6</td>
<td>375 mg/kg</td>
<td>12.63 ± 3.1**(c)</td>
<td>16.34 ± 3.7**(b)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₄</td>
<td>300/6</td>
<td>500 mg/kg</td>
<td>6.56 ± 2.1*(c)</td>
<td>8.83 ± 2.2*(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₅</td>
<td>300/6</td>
<td>625 mg/kg</td>
<td>10.21 ± 1.6***(c)</td>
<td>13.68 ± 2.5***(c)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₆</td>
<td>300/6</td>
<td>750 mg/kg</td>
<td>15.74 ± 2.3***(c)</td>
<td>19.45 ± 3.0***(b)</td>
</tr>
<tr>
<td>FeSO₄ + QCT₇</td>
<td>300/6</td>
<td>875 mg/kg</td>
<td>17.64 ± 3.3***(b)</td>
<td>23.14 ± 3.1***(a)</td>
</tr>
</tbody>
</table>

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameters: Tail moment and Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001, Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.005; (c) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey's multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Figure 33 (a): Broken-line relationship of post treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: Tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameter: tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001, Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.005; (c) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation

Figure 33 (b): Broken-line relationship of post treatment of QCT levels with FeSO₄ to reduction in DNA damage in whole blood cells of Wistar rats assessed through comet parameter: Olive Tail moment.

DNA damage induced by iron sulfate and its significant reduction with the different levels of QCT in whole blood cells of Wistar rats assessed through comet parameter: Olive tail moment. Values are expressed as mean ± SD. Statistically significant different compared to control: * P< 0.05; ** P< 0.001, Statistically significant different from FeSO₄ groups: (a) P< 0.05; (b) P< 0.005; (c) P< 0.001, as analyzed by an analysis of variance (ANOVA) followed by Tukey’s multiple comparison test QCT= quercetin; FeSO₄= iron sulfate; SD= standard deviation.
Figure 34. SCGE (Comet) pictures illustrating the effects of iron sulfate and quercetin (QCT) post treatment on the extent of DNA damage. [A] control (DMSO); [B] treated with FeSO₄ at the dose of 200 mg Fe/kg; [C, D, E, F, G, H, I] treated with QCT at the doses of 125, 250, 375, 500, 625, 750 and 875 mg/kg along with FeSO₄ at 200 mg Fe/kg respectively.
Chapter 5
Discussion
DISCUSSION

In the present study, the chromosomal aberration, micronucleus assay and the comet assay were cooperatively used to study the genotoxic effect of iron sulfate and modulatory effects of pre, simultaneous and post treatment of TQ and QCT against iron sulfate induced genotoxicity at a chromosomal level, micronuclei level as well as a DNA level for 24 h. It has been reported that genotoxic chemicals in general produce the highest frequency of aberrations in rodents 24 h after single exposure, which roughly coincides with the normal length (22–24 h) of the cell cycle (Adler, 1984; Hayashi, 1984). Three genotoxicity evaluation methods cover different aspects of genotoxic events. Bone marrow chromosomal aberration is one of the most effective and sensitive short-term in vivo bioassay that utilizes cytological damage as an end-point in detecting and screening chemical agents that induce clastogenicity (Sanchez et al., 1988). Chromosome analysis of bone marrow cells in vivo is a standard method for testing for the potential mutagenic effects (Celik et al., 2003). The micronucleus assay detects chromosomal damage that persists for at least one mitotic cycle, while the comet assay identifies repairable DNA damage or alkali-labile sites (Zhong et al., 2001). Some of the DNA lesions, such as single and double strand breaks, and adduct formation, led to permanent fetal damage, and the micronucleus test is an excellent means of evaluating any permanent damage in genetic material. On the other hand, the comet assay is a very sensitive method for measuring DNA strand breaks at a single cell level and thus is sensitive to acute DNA lesions. Moreover, it was recently shown that the comet assay detected nearly 90% of carcinogens that were negative or equivocal in the micronucleus assay. Therefore, a combination of the chromosomal aberration, micronucleus assay and the comet assay is recommended for the broad assessment of in vivo genotoxic potential (Pfuhler et al., 2007; Kirkland and Speit, 2008). The National Toxicological Program (NTP) is presently using this combined protocol as part of its efforts to evaluate the genotoxicity of substances of public health concern.

In our experiments of first part we used iron sulfate alone since we aimed to determine the concentration at which iron exerts distinct genotoxic effects. When we evaluated the number of chromosomal aberrations in bone marrow using bone marrow chromosomal aberration assay, number of MNPCEs (as genetic marker) in PCEs, in bone marrow
using the micronucleus assay, and the extent of DNA damage in terms of tail moment and olive tail moment in whole blood cells by the Single cell gel electrophoresis (SCGE/Comet assay), the number of chromosomal aberrations, the numbers of micronucleated polychromatic erythrocytes and the extent of DNA damage increased with increase in the concentration of iron sulfate.

Metal ions can generate DNA damage directly or induce the formation of reactive oxygen species (ROS), leading to DNA damage indirectly probably via Fenton-like reactions (Linder, 2001; De Freitas and Meneghini, 2001). Recently, we observed clastogenicity after acute exposure (24, 48 and 72 h) of human lymphocyte culture to iron sulfate in vitro as evaluated by the chromatid and chromosomal type aberrations (Parveen and Shadab, 2011). In addition, Pagano et al. (1996) demonstrated a high mutagenic and teratogenic potential of bauxite factory samples, where high levels of metals are found, including Fe. Low concentrations of Fe are not able to induce genotoxic effects, since this metal is a common chemical element of cells, and is essential for organisms as a co-factor in oxygen transport (Ganong, 1993; Nelson and Cox, 2002). However, at high concentrations, this metal presents a significant risk for the development of population disorders. Neoplasias and cardiac, pulmonary, hepatic, gastrointestinal and renal alterations are related to Fe exposure. This metal is also toxic to neural tissue and it is related to an increased risk for the development of neurodegenerative disorders (Chau et al., 1993; Lima, 2001).

In our study, as expected, there was a significant difference in the number of chromosomal aberrations, number of MNPCes and DNA damage produced between the lowest (50 mgFe/kg) and highest concentrations (200 mgFe/kg) of iron sulfate. High levels of chromosomal and chromatid aberrations were found in human lymphocytes and TK6 lymphoblast cells exposed to high-energy iron ions (56Fe) (Evans et al., 2001; Durante et al., 2002; Evans et al., 2003). Glei et al. (2002) detected a significant DNA damage, determined by micro gel electrophoresis, in differentiated human colon tumor cells (HT29 clone 19A) incubated with ferric-nitritotriacetate (Fe-NTA). Mutagenic activity was also found in elemental and salt forms of Fe, evaluated with the tests for mutagenicity in Salmonella typhimurium and L5178Y mouse lymphoma cells (Dunkel et al., 1999). It has also been reported that iron compounds are mutagenic in cultured mammalian cells, as detected by Syrian hamster embryo cell transformation/viral
enhancement assay (Heidelberger et al., 1983), sister chromatid exchange (SCE) in hamster cells (Tucker et al., 1993) and base tautomerization in rat hepatocyte cultures (Abalea et al., 1999). The MNPCES in young erythrocytes arise primarily from chromosome fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells and changes in the incidence of MNPCES are considered to reflect chromosomal damage (Salamone and Heddle, 1983). The present study also reveals that high dose of iron increases the number of MNPCES in the polychromatic erythrocytes. In support of this in recent study the results show significant genotoxicity for iron at the high doses tested (Pra et al., 2008). Our comet assay results confirm that there is a significant increase in the extent of DNA damage in terms of tail moment and olive tail moment by the administration of iron sulfate in dose dependent manner. Many ideas about the physiology of metals have being questioned and there is growing evidence that copper and iron might be associated to DNA through in situ reactions, leading to genome damage (Tkeshelashvili et al., 1991; Meneghini, 1997).

Present study indicated that high dose of iron increases the number of chromosomal aberrations, MNPCES, and the extent of DNA damage in dose dependent manner. Oxidative damage is among the most potent and omnipresent threat faced by any living organism. Intracellular accumulation of reactive oxygen species can arise from toxic insults and can perturb the cell's natural antioxidant defense system resulting in damage to all major classes of biological macromolecules. Therefore, arising attention is given to the study of natural products, which may counteract the detrimental effects of oxidative stress and prevent multiple human diseases. In this line, different types of fruits and vegetables have been re-evaluated and recognized as valuable sources of nutraceuticals. Generally, antioxidant and antimutagenic effects of medicinal plant extracts or their constituents are evaluated in vitro applying bioassays such as Salmonella typhimurium (Ferrer et al., 2001) or bone marrow cells, spleenocytes and human lymphocytes (Aboul-Ela, 2002). TQ is the bioactive constituent of the volatile oil of black seed (El-Dakhakhany, 1963). It exerts antioxidant effects and inhibits inflammation in animal models and cell culture systems. It is widely accepted that antioxidants, either endogenous or from the diet, play a key role in preserving health. They are able to quench radical species generated in situations of oxidative stress, either triggered by pathologies or xenobiotics, and they protect the integrity of DNA from genotoxicants. To
examine this issue, our present work was initiated to determine whether TQ and QCT protect the cells against the genotoxicity of iron. Different doses of TQ along with iron were found very effective in reducing the chromosomal aberrations, number of MNPCEs and DNA damage. The protective effects of TQ are likely due to its antioxidant properties (Mansour et al., 2002). Iron excess is responsible to generate oxidative stress, and increases the steady state dose of reactive oxygen and nitrogen species. The toxicity of superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) arises from their iron dependent conversion into the extremely reactive hydroxyl radical (OH-) (Haber–Weiss reaction) that causes severe damage to membranes, proteins, and DNA (Halliwell and Gutteridge, 1984). Consequences include enhancement of radio sensitivity, mutation, lipid peroxidation, polysaccharide depolymerization, enzyme inactivation, degenerative aging and cell death (Weinberg and Miklossy, 2008). The present investigation demonstrated ameliorative effects of pre, simultaneous and post treatment of TQ against genotoxicity induced by iron sulfate. Accidental 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 between 20 and 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). The dose of iron sulfate used in this study was in the higher limit of this range in rat model.

Our experiments showed that treatment with acute ferrous sulfate (200 mgFe/kg) significantly induced an increase in the number of chromosomal aberrations in the bone marrow cells of rats ($P<0.001$). Bone marrow cells are extremely susceptible to oxidative damage and sensitive to clastogenic chemicals (Umegaki et al., 1997). In contrast, of the seven doses of TQ, the highest 21 and 24 mg/kg appeared to be less efficient in decreasing the total number of chromosomal type aberrations in animals treated with iron sulfate 200 mg/kg. These results are in line with previous reports which showed that high doses of TQ cause depletion of cellular glutathione (Badary et al., 1998). Furthermore, TQ caused dose dependent genotoxic effects in Balb/c liver and kidney; it develops an increase of the frequency of chromosomal aberration and the DNA damage index. The increase of CAT is significant at 80 mg/kg. Similarly, the increase of the DNA damage index is significant at the highest concentration used (80 mg/kg) (Harzallah et al., 2012).
It is acknowledged that an increase in the frequency of chromosomal aberrations in bone marrow cells and consequently in peripheral blood lymphocytes is associated with an increased overall risk of cancer (Hagmar et. al., 1994). Most of the chromosomal aberrations observed in the cells are lethal, but there are many other aberrations that are viable and cause genetic effects, either somatic or inherited (Swierenga et. al., 1991). However in the present investigation a significant reduction was observed with pre, simultaneous and post treatment of TQ at the doses of 6, 9, 12, 15 and 18 mg/kg but TQ was found statistically most efficient in reducing the chromosomal aberrations at the dose of 18 mg/kg during simultaneous treatment. Although in one study, the best response in reducing serum levels of hepatic enzymes was observed at the dose of 9 mg/kg. Higher doses of TQ (18 mg/kg), however, provided lesser hepatoprotection (Nili-Ahmadabadi et. al., 2011). Many studies reported cytoprotective activity of TQ against the selective tissue toxicity induced by standard chemotherapeutic agents (Al-Shabanah et al., 1998; Badary, 1999; Badary et al., 1997, 2000). It is likely that the chemopreventive properties of TQ may be related to its antioxidant activity (Houghton et. al., 1995; Burits and Bucar, 2000). Comet assay results also showed that, the increased TM induced by iron sulfate at 200 mg Fe/kg could be reduced by pre, simultaneous and post treatment with TQ at the doses of 6, 9, 12, 15 and 18 mg/kg but significant decrease in DNA damage in terms of decreased TM was observed at 18 mg/kg dose of TQ in comparison with negative control (P<0.001). On the other hand, pre, simultaneous and post treatment with TQ at the doses of 6, 9, 12, 15 and 18 mg/kg decreased OTM induced by Iron sulfate at 200 mg Fe/kg, but significant decrease in the OTM was observed at 18 mg/kg dose of TQ in comparison with negative control (P<0.001). Another study reported that with a dose of 5 mg/kg, TQ had a potent preventive effect on calculus formation and a highly disruptive effect on CaOx kidney calculi. They also tried 10 mg/ kg of TQ and documented its potent preventive effect on kidney calculus formation and a moderate effect on disruption of the kidney calculi. These data suggest that lower doses of TQ might be more effective on the treatment of CaOx kidney calculi. This is in accordance with our findings. Intramuscular administration of TQ into rats in doses of 4 mg/kg/day for four days stimulated the excretion of uric acid in urine. Furthermore, oral administration of TQ to both rats and mice in doses of 4-5 mg/kg for several days protected the animals against cisplatin induced nephrotoxicity (Badary et. al., 1997). In another study, Marozzi et. al. (1970)
reported a TQ-induced enhancement of histamine in induction of gastric ulcers, yet Kanter et al. (2005) reported that oral administration of TQ at a dose of 10 mg/kg one hour before oral absolute alcohol at a dose of 4 ml/kg to rats protected the animals against alcohol-induced ulcers by approximately 38% via antioxidant mechanisms that involved inhibition of reactive oxygen radicals and an increase in superoxide dismutase availability. In this respect the effect of TQ was less than that of the whole volatile oil. Present investigation demonstrated that at the dose of 21 and 24 mg/kg TQ could not show protective role against DNA damage in terms of TM and OTM in all treatments, which may be assigned to its pro-oxidant property at higher doses. In a study on the effect of TQ on lipid profile of rats, Bamosa and colleagues tested intraperitoneal injection of TQ with varying doses of 0.4 mg/kg to 8 mg/kg. They found that there was no linear association of the dose and lowering effect of TQ on serum lipids. The highest dose they used (8 mg/kg) had toxic effects (Bamosa et al., 2002). Recently, Badary et al. (2003) reported that the antioxidant activity of TQ is mainly due to its potent superoxide scavenging potential. It seems reasonable to postulate that TQ exerts its anticlastogenic activity by suppressing ROS generated during the metabolic activity of iron sulfate and hence protects against DNA damage.

TQ has 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 at the dose of 18 mg/kg during simultaneous treatment. Based on the broken line regression analysis of chromosomal aberrations, micronucleus and DNA damage data; it appears that the optimum level of TQ at which it shows its maximum protective effects against genotoxicity induced by iron sulfate is 18 mg/kg.

From the above discussion it is clearly stated that iron has the potential to cause significant oxidative damage leading to mutagenesis (Loeb et. al., 1988). The precise mechanism of iron induced oxidative damage to DNA is not known but is believed to involve free iron that catalyzes the formation of hydroxyl radicals by the Fenton reaction (Nair et. al., 1999). DNA damage may result directly by the oxidation of nucleoside bases or indirectly via the formation of lipid peroxides. To mitigate such oxidative DNA damage, flavonoids have been identified as fulfilling most of the criteria to be considered as antioxidants: the flavonoids inhibit the enzymes responsible for O2·− production (Hanasaki et. al., 1994; Ursini et. al., 1994); the low redox potentials of flavonoids
thermodynamically allow them to reduce highly oxidising free radicals such as $O_2^-$, RO' and HO' (Buettner, 1993); and a number of flavonoids chelate trace metals (Pietta, 2000). Besides scavenging, flavonoids may stabilise free radicals by complexing with them (Shahidi and Wanasundara, 1992). In vitro and animal studies have confirmed that flavonoids possess anti-inflammatory, antioxidant, antiallergenic, hepato-protective, antithrombotic, antiviral and anticarcinogenic activities (Middleton et. al., 2000).

We chose to study QCT because of its superior antioxidant effect among other flavonoids. An extensive amount of in vitro and in vivo animal research has focused on the antioxidant potential of QCT. (De Whalley et. al., 1990; Skaper et. al., 1997; Huk et. al., 1998; Shoskes, 1998; Mojzis et. al., 2001; Inal et. al., 2002; Kahraman et. al., 2003; Su et. al., 2003; Meyers et. al., 2008; Das et. al., 2008; Senfi et. al., 2009; Annapurna et. al., 2009; Hwang et. al., 2009; Lu et. al., 2010; Park et. al., 2010; Tota et. al., 2010; Yao et. al., 2010). Animal evidence suggests QCT's antioxidant effects afford protection of the brain, heart, and other tissues against ischemia-reperfusion injury, toxic compounds, and other factors that can induce oxidative stress. Some animal studies have looked at the anti-tumor properties of QCT. In one study, mice were inoculated with ascites tumor cells and then treated intraperitoneally with either QCT or its glycoside, rutin. Animals treated daily with 40 mg/kg QCT had a 20- percent increase in life span, while those treated with 160 mg/kg rutin had a 50% increase in life span. If the rutin treatment was split into two 80 mg/kg treatments per day, the increase in life span became 94% (Ferry et. al., 1996). Another animal study looked at the effect of QCT on mice bearing abdominal tumors derived from a human pharyngeal squamous cell carcinoma line. The mice were given a daily intraperitoneal injection of QCT. All doses tested (20, 200, 400, and 800 mg/kg) demonstrated significant inhibition of tumor growth (Yokoo and Kitamura, 1997). In vivo tests with mouse with 4T1 breast cancer cells showed that QCT suppressed tumor growth and prolonged survival. QCT enhanced therapeutic efficacy of doxorubicin and reduced toxic side effects. Another Chinese study by Shan and Wang of the Hebei Medical University, investigated the effects of QCT on the growth of the colon carcinoma cells and the regulation effect of QCT on the Wnt/beta-catenin signaling pathway (Shan et. al., 2009). Furthermore, QCT is known as an excellent metal chelator. Recently, it was confirmed that both anti-radical and chelating effects are involved in the protective effect of QCT (Cheng and Breen, 2000). When QCT was administered orally,
it was poorly absorbed from the digestive tract and did not have a great influence on the organs (Murota and Terao, 2003). QCT is the major flavonoid in the human diet and its daily intake with foods is estimated to be 50-500 mg (Deschner et al., 1991). The protective effect of QCT may also be accounted for, at least in part, by their ability to enhance the activity of a variety of detoxification enzymes and/or to shift the metabolic profile of carcinogens such that the intra concentration of the reactive metabolites is diminished. The precise mechanism of the protective action of QCT on iron mediated cytotoxicity is assumed to be due to the scavenging of superoxide anions that produce hydroxyl radicals via the Haber-Weiss reaction, or by the chelation of metal ions that are used to produce highly toxic hydroxyl radicals from H$_2$O$_2$ via the Fenton reaction. We thus set out to observe the optimum level of QCT, at which it exhibits maximum protection against free radical induced damages due to iron sulfate toxicity in rat bone marrow cells by using chromosomal aberrations, micronucleus assay and in whole blood cells of Wistar rats by using single cell gel electrophoresis for 24 h. The precise mechanism of the protective action of QCT on iron mediated cytotoxicity is due to the scavenging of superoxide anions that produce highly toxic hydroxyl radicals via the Haber-Weiss reaction. This is based on the fact that like other polyphenolics, the biological effects of QCT are generally attributed to its antioxidative activities in scavenging ROS and chelating irons. Chromosome aberrations induced by iron sulfate (200 mg/kg) decreased markedly in the pre-treatment, simultaneous and post-treatment with QCT, except the three high doses (625, 750 and 875 mg/kg doses of QCT). Like other antioxidant, QCT might have pro-oxidant activity, at least under some circumstances. Long-term feeding of QCT (20 mg/day) to Sprague-Dawley rats increased serum and liver alpha-tocopherol concentrations and significantly decreased malondialdehyde concentrations, but also significantly decreased GSH concentrations and glutathione reductase activity (Choi et al., 2005). Our study also indicates that QCT show cytotoxicity as there is increase in chromosomal aberration at the three high doses. However, the cytotoxicity induced by iron sulfate was found to be ablated by QCT. This indicates that QCT itself can induce dose dependent genotoxic and cytotoxic effect at high doses but the iron sulfate induced damages were reduced by it. Several pharmacological and biological properties that may be beneficial to the health of humans, including among others antioxidant, anti-inflammatory, and anti-carcinogenic characteristics, have been ascribed to QCT (Middleton et al., 2000); however, the
antioxidant and free-radical scavenging properties of QCT and similar compounds, which are generally regarded as positive functions, also have been linked to less desirable pro-oxidant activities (Metodiewa et al., 1999; Boots et al., 2003).

Unlike the positive results obtained in vitro and in vivo following intraperitoneal dosing, tested in vivo in animal oral administration studies, QCT has not demonstrated any genotoxic activities. The results from the current series of in vivo genotoxicity studies conform with previous investigations of potential in vivo genotoxicity, which demonstrated an absence of positive activity when various relevant endpoints (i.e., MN, chromosomal aberrations, sister chromatid exchange, UDS, and alkali-labile DNA damage) in somatic cells obtained from animals treated orally with QCT were compared with those from negative controls (MacGregor, 1979; Aeschbacher et al., 1982; Ishikawa et al., 1985; Ngomuo and Jones, 1996; Taj and Nagarajan, 1996; Cierniak et al., 2004). An abundance of in vitro and in vivo animal experiments have attempted to elucidate QCT's effect in cancer. In vitro evidence indicates that QCT has a variety of anticancer mechanisms, including antioxidant, antiproliferative, pro-apoptotic, cell signaling effects, and growth factor suppression, as well as potential synergism with some chemotherapeutic agents (Yoshida et al., 1990; Scambia et al., 1992; Larocca et al., 1995; Singhal et al., 1995; Richter et al., 1999; ElAttar and Virji, 1999; Caltagirone et al., 2000; Zhang et al., 2000; Ranelletti et al., 2000; Haggiac and Walle, 2005; Braganhol et al., 2006; Dhal et al., 2006; Rosner et al., 2006; Granado-Serrano et al., 2006; Gulati et al., 2006; Conklin et al., 2007; Hung, 2007; Aalinkeel et al., 2008; Ramos and Aller, 2008; Choi et al., 2008; Galluzzo et al., 2009; Jeong et al., 2009; Jung et al., 2009; Kawahara et al., 2009; Borska et al., 2010; Linsalata et al., 2010; Olson et al., 2010; Vidya Priyadarsini et al., 2010). QCT has also been shown to inhibit the growth of cancer in vivo in animal experiments designed to promote tumor formation (Castillo et al., 1989; Deschner et al., 1991; Pereira et al., 1996; Matsukawa et al., 1997; Yang et al., 2000; Ferrer et al., 2005; Jin et al., 2006; Choi et al., 2006; Warren et al., 2009). While most of the animal studies have shown a beneficial effect (especially in preventing colon tumorigenesis), a high dose of QCT did not prevent UVB-induced carcinogenesis. (Steerenberg et al., 1997) A modified QCT (QCT chalcone) and a pH-modified citrus pectin were reported to reduce the growth of solid primary tumors (Hayashi et al., 2000).
In the present study reported here, chromosome aberrations induced by iron sulfate (200 mg/kg) decreased markedly in the pre-treatment, simultaneous and post-treatment with QCT at the doses of 125, 250, 375 and 500 mg/kg. In agreement with previous reports the multiple protective activities of QCT arise from its strong antioxidant properties (Scambia et al., 1991; Elattar and Virji, 2000; Caltagirone et al., 2000; Aligianni et al., 2001). Its antioxidant effect was documented in many in vitro and in vivo experimental studies (Mojzis et al., 2001). It has been recently observed that QCT may behave as a cytotoxic agent and as a mutagen at much higher doses (Sahu and Washington, 1991). Thus, the ineffectiveness of QCT at higher doses has been attributed to its prooxidant activities (Yoshino et al., 1999; Metodiewa et al., 1999). In the present study we also observed these apparently contradictory activities of QCT that it can act as both antioxidant and prooxidant, depending on concentration and the source of the free radicals (Laughton et al., 1989; Sugihar et al., 1999). In one study, protection was more prominent in 200 mg/kg as compared to 100 mg/kg. This protection might be due to the quenching of lipid peroxides by QCT from the cells. QCT also donates an electron to the free radicals formed in the body (Molina et al., 2003). Among pre, simultaneous and post treatments, the protective effect of QCT was most prominent in the simultaneous treatment and the best response was observed at 500 mg/kg. Pre, simultaneous and post treatments of QCT reduced the number of MNPCEs induced by iron sulfate at the doses of 125, 250, 375 and 500 mg/kg. The results suggest that QCT reduced the number of MNPCEs induced by iron sulfate, but it could not completely protect cells from damage. The most efficient antielastogenic effect of QCT was observed in 500 mg/kg dose, at which it shows maximum protective effect against genotoxicity and elastogenicity induced by iron sulfate.

We observed significantly lower effect for both DNA damage in terms of tail moment and olive tail moment when QCT was administered through the i.p. route at the doses of 625, 750 and 875 mg/kg. In contrary to the present study another animal study looked at the effect of QCT on mice bearing abdominal tumors derived from a human pharyngeal squamous cell carcinoma line. The mice were given a daily intraperitoneal injection of QCT. All doses tested (20, 200, 400, and 800 mg/kg) demonstrated significant inhibition of tumor growth. The 20 mg/kg dose had an effect only slightly less than that seen with 800 mg/kg. The effect on the growth of normal human fibroblast cells was
minimal. The author concluded that QCT appears to be a selective inhibitor of tumor cell growth (Castillo et al., 1989). An effectively significant protection by pre, simultaneous and post treatment of QCT against the formation of oxidative DNA damage generated by iron sulfate was observed at the doses of 125, 250, 375 and 500 mg/kg. Two animal studies have looked at the anti-tumor properties of QCT. In one study, mice were inoculated with ascites tumor cells and then treated intraperitoneally with either QCT or its glycoside, rutin. Animals treated daily with 40 mg/kg QCT had a 20-percent increase in life span, while those treated with 160 mg/kg rutin had a 50-percent increase in life span. If the rutin treatment was split into two 80 mg/kg treatments per day, the increase in life span became 94 percent. These in vivo results are interesting since in vitro work showed rutin to have little effect compared to QCT on tumor tissue (Kuo, 1996; Larocca et al., 1991). Human studies have not shown any adverse effects associated with oral administration of QCT in a single dose of up to four grams or after one month of 500 mg twice daily (Shoskes et al., 1999). QCT may also have anti-mutagenic properties. A group of scientists lead by Gupta of the National Institute of Pharmaceutical Education and Research, Mohali, India, found that QCT may be a potential candidate as chemoprotectant. They came to this conclusion after treating rats, which were exposed to the hepatocarcinogen diethylnitrosamine (found in tobacco smoke and processed meat) with QCT. The hepatocarcinogen increased malondialdehyde and decreased glutathione levels in the liver, and increased plasma levels of aspartate transaminase and alanine transaminase. Treatment of the rats with QCT restored these levels and also reduced diethylnitrosamine induced DNA damage and apoptosis (Gupta et al., 2009). Protection against H₂O₂ was confirmed for myricetin, QCT and rutin in Caco-2 and HepG2 cells (O'Brien et al., 2000) and for QCT and luteolin in murine and human leukaemia cell lines (Horvathova et al., 2003; 2004). With regard to other genotoxicants, QCT and rutin displayed antigenotoxic effects on DNA damage induced by mitomycin C, in a concentration-dependent manner (Undeger et al., 2004). This study confirmed the antigenotoxic and protective effect of QCT and the best result was observed at the dose of 500 mg/kg QCT in simultaneous treatment. However, the mild prooxidant activity of QCT was observed at the high doses (625, 750 and 875 mg/kg). One possible mechanism for the protection against cyto- and genotoxicity may be that simultaneous treatment with QCT would allow interception of free radicals generated by iron sulfate before they reach DNA and induce cyto- and genotoxicity. In the present study we found QCT as a
strong inhibitor of iron-dependent OH· formation which helped in protecting against OH·-induced chromosomal aberrations, MNPCEs and DNA damage in bone marrow and blood cells of Wistar rats. Further studies are required to elucidate the real nature and mechanism of the interactions of iron and QCT.

The protective action of TQ and QCT observed here suggests that they have antioxidative properties 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 at the dose of 18 mg/kg and 500 mg/kg during simultaneous treatment.

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. Based on these facts, the intake of this metal must be very carefully regulated.

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, number 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.
Bibliography
REFERENCES


References


130


References


References


Halder, B., Pramanick, S., Mukhopadhyay, S. and Giri, A.K., 2005. Inhibition of benzo[a]pyrene induced mutagenicity and genotoxicity by black tea


References


References


the comet assay as an alternative to in vitro clastogenicity tests for early drug

Evaluation of bioflavonoids as potential chemotherapeutic agents. Biomed Sci

World Health Organization (WHO), 1998. Trace elements in human nutrition and
health.

Wurzelmann, J.I., Silver, A., Schreinemachers, D.M., Sandler, R.S. and Everson,

Yamagishi, M., Osakabe, N., Natsume, M., Adachi, T., Takizawa, T., Kumon, H. and
liquor polyphenols against mitomycin C-induced DNA damage. Food Chem
Toxicol, 39(12): 1279-83.

Yang, K., Lamprecht, S.A., Liu, Y., Shinozaki, H., Fan, K., Leung, D., Newmark, H.,
Steele, V.E., Kellogg, G.J. and Lipkin, M., 2000. Chemoprevention studies of
the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse
colon. Carcinogenesis, 21(9): 1655-60.

Yi, T., Cho, S.G., Yi, Z., Pang, X., Rodriguez, M., Wang, Y., Sethi, G., Aggarwal,
B.B. and Liu, M., 2008. Thymoquinone inhibits tumor angiogenesis and tumor
growth through suppressing AKT and extracellular signal-regulated kinase
signaling pathways. Mol Cancer Ther, 7(7): 1789-96.

cells from oxidant triggered apoptosis by bioflavonoid quercetin. Am J
Physiol, 273: 206-12.

Yoshida, M., Sakai, T., Hosokawa, N., Marui, N., Matsumoto, K., Fujioka, A.,

Yoshimoto, T., Furukawa, M., Yamamoto, S., Horie, T. and Watanabe-Kohno, S.,


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄</td>
<td>Iron Sulfate</td>
</tr>
<tr>
<td>TQ</td>
<td>Thymoquinone</td>
</tr>
<tr>
<td>QCT</td>
<td>Quercetin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>CAAs</td>
<td>Chromosomal Aberrations</td>
</tr>
<tr>
<td>MNT</td>
<td>Micronucleus Test</td>
</tr>
<tr>
<td>PCEs</td>
<td>Polychromatic Erythrocytes</td>
</tr>
<tr>
<td>MNPCES</td>
<td>Micronucleated Polychromatic Erythrocytes</td>
</tr>
<tr>
<td>NCEs</td>
<td>Normochromatic Erythrocytes</td>
</tr>
<tr>
<td>TM</td>
<td>Tail Moment</td>
</tr>
<tr>
<td>OTM</td>
<td>Olive Tail Moment</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>gm</td>
<td>Gram</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>bw</td>
<td>Body Weight</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>p.o.</td>
<td>Orally</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>Disodium Ethylenediaminetraacetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>LMP</td>
<td>Low Melting Point Agarose</td>
</tr>
<tr>
<td>DDW</td>
<td>Double Distilled Water</td>
</tr>
</tbody>
</table>

156
REAGENTS FOR CHROMOSOMAL ABERRATIONS

Hypotonic solution KCl

0.075 M KCl (potassium chloride) solution prewarmed at 37°C was used.

560 mg KCl was dissolved in 100 ml autoclaved DDW.

Colchicine

It is a mitotic poison. It arrests spindle fibers at metaphase stage. Colchicine is a highly poisonous natural product and secondary metabolite, originally extracted from plants of the genus Colchicum. (Autumn crocus, *Colchicum autumnale*, also known as the “Meadow saffron”) Colchicum extract was first described as a treatment for gout in *De Materia Medica* by Pedanius Dioscorides in the first century CE. The colchicine alkaloid was first isolated in 1820 by the two French chemists P.S. Pelletier and J. Caventon. It was later identified as a tricyclic alkaloid, and its pain relieving and anti-inflammatory effects for gout were linked to its ability to bind with tubulin.

Biological function

Colchicine inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to mitosis, and therefore colchicine effectively functions as a “mitotic poison” or spindle poison. 2 mg/kg colchicine was used. It can be stored at 2 to 5°C for several months.

Fixative

Freshly prepared glacial acetic acid: methyl alcohol (1:3) was used.

Giemsa stain

Giemsa stain, named after Gustav Giemsa, an early malarialogist, is used for the histopathological diagnosis of malaria and other parasites. It is a mixture of methylene blue and eosin. The stain is usually prepared from commercially available Giemsa powder. It is specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine-thymine bonding. *Giemsa stain* is used in Giemsa banding, commonly called G- banding, to stain chromosomes and often used to create a karyotype. It can identify chromosomal aberrations such as translocations and interchanges.
Shelf Life and Storage

Giemsa stain solution has a shelf life of 2 years when stored unopened at +15°C - +25°C diluted solution is stable for one day.

Giemsa stain (stock)

1.0 gm Giemsa powder was mixed in 66 ml of glycerine and placed in 60°C over for 24 h (one day) and then methanol is added.

a. Working solution 1

1.25 ml of Giemsa stock is added in 1.5 ml of methanol in 50 ml of autoclaved distilled water.

b. Working solution 2

5 ml of Giemsa stock is added in 45 ml of phosphate buffer solution.

**REAGENTS FOR MICRONUCLEUS TEST**

May-Grunwald’s Stain

**Solution 1.** Dissolve 125 mg May-Grunwald’s dye in 100 ml methanol and filter the solution.

**Solution 2.** 50 ml May-Grunwald’s solution 1 + 50 ml DDW.

**REAGENT PREPARATION FOR SINGLE CELL GEL ELECTROPHORESIS ASSAY (COMET ASSAY)**

Stock solution

Saline (0.9%)

0.9 gm NaCl was dissolved in 100 ml DDW.

5 M NaCl

146.1 gm NaCl was dissolved in 500 ml DDW to make 5M NaCl.
500mM Na\textsubscript{2}EDTA (pH= 8)

93.06 gm Na\textsubscript{2}EDTA was dissolved in 500 ml DDW to make 0.5 M solution (dissolve by adding NaOH pallets).

**Tris buffer (pH= 7.5)**

36.342 gm tris was dissolved in 100 ml DDW to make 3M solution.

**3 M NaOH**

60 gm NaOH was dissolved in 500 ml DDW to make 3M solution.

**Working solution**

**LMP Agarose (0.8%)**

8 mg LMP was dissolved in 1 ml 0.9% saline solution.

**Lysis Buffer (500 ml)**

100 ml 0.5 M EDTA + 250 ml 5M NaCl + 50 ml DMSO 10% + 5 ml Triton-x (1%) were dissolved to make up to 500 ml with autoclaved DDW. Lysis buffer removes non nuclear components from the cells.

**Alkaline buffer (800 ml) (pH= 13)**

Stock 3M NaOH.................Working 0.3M= 80 ml

Stock 0.5M EDTA.................Working 1mM= 1.6ml

To make final volume up to 800 ml with autoclaved DDW.

Alkaline buffer allows DNA unwinding and conversable of alkali labile sites to single stranded breaks.

NaOH...3M x V = 0.3 x 800/30 = 80 ml

EDTA...500Mm x V = 1mM x 800/500 = 1.6 ml.

**Tris buffer (240 ml) (pH=7.5)**

Stock 3M Tris...............Working 0.4M= 48ml

2 M x V = 0.4 M x 240 ml

V= 0.4 x 240/2= 48 ml.

Taken 48 ml Tris make final volume up to 240 ml with autoclaved DDW. It is used for remove alkali and detergents. Thus neutralize the pH of cells as it is acidic.