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 \textit{in vitro} and \textit{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 \textit{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 \textit{in vitro} and \textit{in vivo} tests have been developed for genotoxic screening of chemicals. The most commonly used \textit{in vivo} conditions are several transgenic animal models to detect chemicals causing gene mutations, and the \textit{in vivo} micronucleus test and the \textit{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>
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<tr>
<td>Pre mutagenic lesions</td>
<td>Interaction of chemical and DNA</td>
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<td>DNA damage</td>
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<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>
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### 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.

![Figure 2. Iron Distribution in the Adult Human Body.](image)

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 constrains 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 (OCl$^-$), 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 peroxy (ROO$^.$), alkoxy (RO$^.$), thyl (RS), or thylperoxyl (RSOO$^.$) radicals (Figure 3B).
A.

\[
\text{Fe (II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe (III)} + \text{OH}^- + \text{OH}^+ \quad \text{(Fenton)}
\]

\[
\text{Fe (III)} + \text{O}_2^- \rightarrow \text{Fe (II)} + \text{O}_2
\]

net reaction

\[
\text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \text{OH}^- + \text{OH}^+ + \text{O}_2 \quad \text{(Haber-Weiss)}
\]

B.

\[
\text{Fe (II)} + \text{ROOH} \rightarrow \text{Fe (III)} + \text{OH}^- + \text{RO}^-
\]

\[
\text{Fe (III)} + \text{ROOH} \rightarrow \text{Fe (II)} + \text{H}^+ + \text{ROO}^-
\]

\[
\text{RSH} + \text{OH}^- \rightarrow \text{RS}^- + \text{H}_2\text{O}
\]

\[
\text{RSH} + \text{ROO}^- \rightarrow \text{RS}^- + \text{ROOH}
\]

\[
\text{RS}^- + \text{O}_2 \rightarrow \text{ROO}^-
\]

C.

\[
\text{Heme-Fe (II)-O}_2 + \text{H}_2\text{O}_2 \rightarrow \text{Heme-Fe(IV)- OH}^- + \text{O}_2 + \text{OH}^-
\]

\[
\text{Heme-Fe (IV)- OH}^- + \text{ROOH} \rightarrow \text{Heme-Fe(III)+ ROO}^- + \text{H}_2\text{O}_2
\]

D.

\[
\text{Fe (II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe (II)-O} + \text{H}_2\text{O}
\]

\[
\text{Fe (II)} + \text{O}_2 \rightarrow [\text{Fe (II)-O}_2 \rightarrow \text{Fe (III)-O}_2^-] \rightarrow \text{Fe (III)+ O}_2^-
\]

**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 Pantapoulos, 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 \([\text{O}_2]/[\text{H}_2\text{O}_2]>100\), 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.

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-nitrilotriacetate (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, elastogenic 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 Ghio, 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 antimutagenic 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 antimutagenic 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).

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

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.](image)

**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-butylhydroquinone (TBHQ), TQ was found to be more effective than TBHQ as a superoxide anion scavenger, but not as a scavenger of 2, 20-diphenyl-picrylhydrazyl 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).
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 [SCCvII; (Ivankovic et. al., 2006)], fibrosarcoma [FSSaR; (Ivankovic et. al., 2006)], laryngeal neoplastic cells-Hep-2 (Womack 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 proteasomal 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

(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).

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

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).
Figure 12. A scheme showing free radicals and their relationship to body injuries induced by iron sulfate and the potential protective role of quercetin.

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.
Figure 14. QCT targets different sites in inflammatory diseases and cancer

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 \textit{in vitro} and \textit{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 (Soobrattee \textit{et al.}, 2006).

In a study done by Caltagirone \textit{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 (Heloísa \textit{et al.}, 2004). Furthermore QCT seems to inhibit angiogenesis (Fotsis \textit{et al.}, 1997). Angiogenesis is normally a strictly controlled process in the human body. Pathological, unregulated angiogenesis occurs in cancers (Fan \textit{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 \textit{et al.}, (1995) stated that regular consumption of flavonoids in the food might reduce the risk of deaths from CHD in elderly men (Hertog \textit{et al.}, 1993; Hertog \textit{et al.}, 1995). QCT was also shown to be effective inhibitor of platelets aggregation in dogs and monkeys (Osman \textit{et al.}, 1998). The main antiplatelet aggregating effect is because of the inhibition of thromboxane A2 (Tzeng \textit{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 \textit{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 \textit{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 (Alarcon 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 prostodynia). 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.