Green tea activity and iron overload induced molecular fibrogenesis of rat liver

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Abstract
Iron overload toxicity was shown to associate with chronic liver diseases which lead to hepatic fibrosis and subsequently the progression to cancer through oxidative stress and apoptotic pathways. Green tea potential activity as chelating, anti-oxidative, or anti-apoptotic mechanisms against metal toxicity was poorly clarified. Here, we are trying to evaluate the anti-oxidant and anti-apoptotic properties of green tea in the regulation of serum hepcidin levels, reduction in iron overloads, and improve of liver fibrosis in iron overloaded experimental rats. Three groups of male adult rats were randomly classified into three groups and treated as follows: control rats, iron treated rats for two months in drinking water followed by either vehicle or green tea extract (AGTE; 100 mg/kg) treatment for 2 more months. Thereafter, we studied the effects of AGTE on iron overload-induced lipid peroxidation, anti-oxidant depletion, liver cell injury and apoptosis. Treatment of iron-overloaded rats with AGTE resulted in marked decreases in iron accumulation within liver, depletion in serum ferritin, and hepcidin levels. Iron-overloaded rats had significant increase in malonyldialdehyde (MDA), a marker of lipid peroxidation and nitric oxide (NO) in liver when compared to control group. Also, significant change in cytochrome c and DNA content as apoptotic markers were reported in iron treated rats. The effects of iron overload on lipid peroxidation, NO levels, cytochrome c and DNA content were significantly reduced by the intervention treatment with AGTE (P < 0.001). Furthermore, the endogenous anti-oxidant capacities/levels (TAC) in liver were also significantly decreased in chronic iron overload and administration of AGTE restored the decrease in the hepatic antioxidant activities/levels. Also, hepatic hepcidin was shown to be significantly correlated with oxidative and apoptotic relating biomarkers as well as an improvement in liver fibrosis of iron treated rats following AGTE treatment. In-vitro analysis showed that, the improvement in iron toxicity of the liver depend mainly on antioxidant and protective ability of green tea polyphenolic compounds especiallyepigallocatechin-3-gallate (EGCG). Our study showed that green tea extract (GTE) ameliorates iron overload induced hepatotoxicity, apoptosis and oxidative stress in rat liver via inhibition of hepatic iron accumulation; improve of liver antioxidant capacity, and down regulation of serum hepcidin as well as reduction in the release of apoptotic relating proteins.

1. Introduction
Nutritional deficiency is the main target for iron deficiency in human beings with or without anemia. Thus, for all living cells, both essential roles and toxic actions of iron were reported (Wosten et al., 2000). Also, prolonged and uncontrolled iron administration should be avoided for the potentially cellular damaging effects especially in liver cells (Mollet et al., 2016). Higher epidemic levels were reported as a result of excess iron in living cells.

In human and animal research models, iron overload was shown to be related with hereditary hemochromatosis, thalassemia, and hepatic diseases such as chronic viral hepatitis, alcoholic hepatitis (Deugnier et al., 2008). Iron (Fe) is stored within liver cells in various forms including, Fe containing enzymes, ferritin, hemosiderin, and heme (Jomova and Valko, 2011).

As a result of metal binding capacity of iron to some cellular low molecular weight proteins which act as chelators, excess iron was sequestrated, deposited within liver cells, and inducing liver tissue
damage. The deposition of iron in hepatocytes increasing the risk of developing significant fibrosis, cirrhosis, and subsequently increase the rate of morbidity and mortality (Olynyk et al., 2005). Several research studies reported a significant association between the hepatic iron concentration (HIC), hepatotoxicity, and the prognosis of liver fibrosis (Olynyk et al., 2005).

In iron overload, excess iron promotes the generation of reactive oxygen species which stimulates severe oxidative damage to cellular organelles such as lipids, proteins, and nucleic acids (Siah et al., 2006). Also, the liberated oxidative free radicals trigger hepatic inflammation via production of some proinflammatory cytokines (TNF-α, nuclear factor κB) which participate in the pathogenesis of both acute, chronic liver damage, and even cirrhosis (Uchiyama et al., 2008).

Also, excess iron may have additional toxic effect on mitochondrial membranes. It may initiates an opening of the mitochondrial pores which conducts solutes into the mitochondria and consequently produce more damaging process such as mitochondrial depolarization, uncoupling of oxidative phosphorylation, mitochondrial swelling, and depletion in adenosine triphosphate. This finally leads to the release of proapoptotic proteins, like cytochrome c which resulting in hepatic cell necrosis or apoptosis (Uchiyama et al., 2008; Moon et al., 2010). This significant hepatocyte apoptosis plays a significant role in the progression of hepatic fibrogenesis and carcinogenesis (Kowdley, 2004). Although, many mechanisms were present to discuss hepatocellular injury, the estimate pathways involved in liver cell dysfunction and fibrogenesis remain to be sufficiently elucidated (Hubsher, 2003).

Hepcidin is a small 25-amino acid peptide produced within liver cell and can be estimated easily in tissue, serum or urine samples (Frazer et al., 2002). Most studies reported hepcidin as an iron hormone regulator whereas, deficiency in hepcidin level was observed in cases with iron overload, and that overexpression of hepcidin levels were shown in subjects with severe iron deficiency and anemia (Frazer et al., 2002).

The up and down regulation of hepcidin was shown to be linked with iron disorders and in turn estimates its importance in systemic iron homeostasis. The hyposideremic activity of hepcidin regulates iron levels by inhibiting the intestinal absorption, the release of iron by macrophages, and control of the surface expression of the iron exporter ferroportin. It was shown that hepcidin was able to bind to ferroportin, leading to the internalization and degradation of the iron exporter (Nemeth et al., 2004a,b), thereby decreasing iron availability in the circulation. So, hepcidin may has a pivotal role in hepatic fibrogenesis and severity of liver diseases (Frazer et al., 2002), and could be used as a diagnostic parameter for staging of liver fibrosis (Frazer et al., 2002).

The changes in the expression of hepcidin have been reported in many liver diseases. Lower levels of serum hepcidin/ferritin ratio were reported in iron overloaded chronic hepatitis C patients compared to HBV patients (Tan et al., 2012; Fujita et al., 2007). Also, hepatocyte apoptosis induced by iron overload was shown to be linked with the regulation of hepcidin (Ganz, 2011).

Recently, it was reported that over expression of p53 and Fas antigens as apoptosis inducing proteins in hepatoma cells participates in the regulation of hepcidin. Whereas, up regulation of these proteins has been shown to induce hepcidin gene transcription and conversely down regulation of p53 and Fas antigens resulted in down regulation of hepcidin expression (Li et al., 2013).

Chemically synthesized iron chelators have been proposed for the treatment of many diseases associated with iron overload (Pangjiet al., 2015; Kulprachakarn et al., 2014; Chansiw et al., 2014). However, more adverse effects were represented during the treatment schedules that made many pharmacologists to devote their efforts to study new treatment strategies based on naturally occurred iron chelators of plant origin.

Green tea was reported as one of the most naturally present iron chelators which showed both antioxidant and iron chelation activities in vivo and in vitro experimental models (Patel et al., 2012; Saewong et al., 2010). In iron overloaded experimental models, hepcidin levels were regulated with both drug therapy and naturally occurred iron chelators (Porter et al., 2014; Gu et al., 2013). Although, little is known about the exact regulation mechanisms proposed under these conditions, only green tea alone or in combination with other drugs chelating toxic iron in plasma and tissues, and increasing the levels of hepcidin expression (Kautz et al., 2014; Yun and Vincelette, 2015; Upanan et al., 2015). From the previous data, our hypothesis that green tea as naturally occurred iron chelator of plant origin could ameliorates iron overload toxicity and prevent most iron related diseases especially liver diseases. Thus, in this study, we are trying to evaluate the anti-oxidant and anti-apoptotic properties of green tea in the regulation of serum hepcidin levels, reduction in iron overloads, and improve of liver fibrosis in iron overloaded experimental rats.

2. Materials and methods

2.1. Animals and experimental design

A total of 30 young albino male Sprague Dawley rats (Rattus norvegicus) weighing 120–150 g have been randomly included in this study. The animals have been housed in healthy atmospheric conditions, normal feeding, drinking, and medical care based on the guidelines of the experimental animal care, college of science, King Saud University, Riyadh, Saudi Arabia. The experimental procedures were approved by the Ethics Committee of the Experimental Animal Care Society at King Saud University (Permit Number: PT 1204).

The animals were divided randomly into three groups (n = 10); Control group (rats feed on normal diets without iron), Iron overloaded group (rats feed with iron in a drinking water for two month and then left without treatment for another two month), and AGTE treated group (rats feed with iron in a drinking water for two month then treated with 100 mg/kg/day AGTE suspended in drinking water for another two month).

Iron was added to drinking water in a quantity exceeds the maximum permissible concentration (MPC; Fe2+ is 0.3 mg per liter) for this chemical in Ministry of Health. Thus, rats of control group supplemented only tap water, whereas iron overloaded and AGTE groups provided with drinking water containing 3 mg/L of Fe2+ (using 8.3 mg/L of FeSO4). The dose of AGTE was selected based on previous studies (Upanan et al., 2015; Kim et al., 2009). In most animal studies, a dose range of 50–200 mg/kg body weight AGTE exhibited a good anti-inflammatory and anti-fibrotic activity and seemed to have no adverse effects on human (Saewong et al., 2010; Kim et al., 2009; Ibrahim et al., 2015). After four months, rats were sacrificed under ether anesthesia. Blood and liver tissue samples were collected and subjected for subsequent histological and biochemical analysis. For biochemical analysis in liver tissues, part of the samples was immediately frozen at −80 °C until reused.

2.2. Green tea extracts (GTE)

A microwave cabinet was used for drying freshly harvested green tea leaves (Camellia sinensis) (Upanan et al., 2015). To prepare green tea extract, hot water is used. Epigallocatechin 3-gallate (EGCG) as active constituent was estimated in green tea extract by using HPLC method. The GTE product containing 28% (w/w) EGCG was kept in the dark at −20 °C until studied (Kim et al., 2009; Saewong et al., 2010).
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