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METAL COMPLEXES OF DIETARY FLAVONOIDS: EVALUATION OF RADICAL SCAVENGER PROPERTIES AND PROTECTIVE ACTIVITY AGAINST OXIDATIVE STRESS *IN VIVO*

V.A. KOSTYUK[1]*, A.I. POTAPOVICH[1], T.V. KOSTYUK[1] AND M.G. CHERIAN[2]

[1]Department of Biology, Byelorussian State University, Minsk, Belarus,
[2]Department of Pathology, The University of Western Ontario, London, Canada
* Kostyuk Vladimir, Department of Biology, Byelorussian State University,
220050 Minsk, Belarus Tel: 375 (17) 209 5863 Fax: 375 (17) 212-5535 E.Mail: kostyuk@bsu.by

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Abstract – Interaction of certain flavonoids with transition metals increases their water solubility and leads to the formation of flavonoid-metal complexes, which may act as superoxide dismutase mimics with high scavenger potencies toward superoxide. Effect of serum albumin on stability of flavonoid-metal complexes was studied and complex of rutin with iron (II) was found to be the most stable. The ability of flavonoid metal complexes to catalyze homolytic cleavage of hydrogen peroxide was also studied and rutin iron (II) complex was found to be relatively poor Fenton catalyst. The potential therapeutic benefits of this new antioxidant agent were studied using experimental model of pathological states associated with oxidative stress *in vivo*. Acute hepatic injury in MT-I/MT-II null transgenic mice induced by injections of thioacetamide was used for this purpose. It was found that pretreatment with rutin- iron complex protected against thioacetamide induced hepatotoxicity as observed by a significant reduction in the elevated levels of serum enzymes and partial normalization of GSH/GSSG ratio, glutathione peroxidase II and glutathione reductase activity in mice liver. The results demonstrate that flavonoid-metal complexes possess effective free radical scavenger ability and have potent therapeutic benefits for the treatment of oxidative stress-related diseases and dysfunction.

Key Words: free radicals, oxidative stress, knockout mice**,** liver injury, thioacetamide, metallothionein, flavonoids; rutin; (–)-epicatechin; metal flavonoid complexes; glutathione S-transferases, glutathione peroxidase, glutathione reductase, superoxide dismutase.

INTRODUCTION

A variety of potential health benefits, including reduced risk for coronary artery disease and cancer are thought to be associated with dietary flavonoids [15,17,18]. These effects of flavonoids are generally attributed to their ability to scavenge reactive oxygen and nitrogen species ($O_2^{-\bullet}$, $^{\bullet}OH$, $NO^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$) [8,16,28] and reduce oxidative stress, which may contribute to the progression of many diseases. For these reasons flavonoid-rich diet, supplements and cosmetics are widely recommended for improving health status and prevention of chronic diseases. There are only few studies on the use of flavonoids for the treatment of acute tissue injury protection from chemical toxicity. The limited experimental studies on flavonoids partially are due to their poor water solubility. However, flavonoids can form complexes with transition metal ions [1] and these complexes are more hydrophilic and water-soluble than the corresponding ligands. Moreover, flavonoids metal complexes possess higher scavenger potencies toward superoxide than parent flavonoids and may act as superoxide dismutase mimics [20]. Transition metals also enhance the anti-inflammatory activities of flavonoids and their cytoprotective effects against oxidative injury in isolated cells [2,21,25]. The aim of this study was further evaluation of antiradical properties of metal complexes of dietary flavonoids. From this study rutin-iron (II) complex was selected as effective and the most stable antiradical agent and a protective activity of this compound against oxidative stress was examined in model system *in vivo*.

MATERIALS AND METHODS

Animals

Homozygous MT-KO (129s7/SvEv-Brd-Mt1^{tm1Bri} Mt2^{tm1Bri}) and WT mice (129s3/SvImJ) were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred in the animal facilities at the University of Western Ontario. Animals were housed four per cage and were maintained in a controlled temperature and humidity environment of 12:12-hr light:dark cycles. All animals were fed mouse chow (Harlan Teklad 2018) and water *ad libitum*.

Chemicals

Most of chemicals were purchased from Sigma-Aldrich, glutathione reductase was purchased from Roche Molecular Biochemicals, reduced glutathione (GSH), oxidized glutathione (GSSG), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was obtained from Boehringer Mannheim GmbH, hydrogen peroxide and EDTA from Merck. All other reagents were of analytical grade. Complexes of flavonoids with metals were prepared *in situ* by mixing flavonoids and appropriate salts ($FeSO_4$ $7H_2O$; $Fe_2(SO_4)_3$ $9H_2O$; $CuSO_4$) in water (flavonoid : metal ion ratio was 1:1).

Superoxide-mediated reduction of nitroblue tetrazolium by photochemically reduced riboflavin

Reduction of nitroblue tetrazolium (NBT) was carried out at room temperature (22°C) under fluorescent lighting (20 w, 20 cm). The standard incubation mixture (3.5 ml) contained 6 µM riboflavin, 0.8 mM of N,N,N^1,N^1, -tetramethylethylenediamine (TMEDA) in 0.016 M phosphate buffer (pH 7.8) and 85 µM NBT. After 5 min incubation, the reaction was stopped by the light switching off and the addition of 0.05 ml SOD (1 mg/ml), and absorbance was measured at 550 nm.

Animal Experiments and Sampling of Material

Both male and female knockout mice (10- to 16-weeks-old) were used. Mice were injected intraperitoneally with thioacetamide (125 mg/kg body weight). For testing protection against TAA hepatotoxicity, 20 min before treatment with TAA the mice were injected intraperitoneally with rutin-ferrous complex in dose 0.0125 mmol/kg (7 mg/kg body weight of rutin). Control mice were injected with equivalent volumes of saline instead of flavonoid metal complexes and TAA. At the time points indicated after TAA (saline) injection all mice were euthanized by an overdose injection of pentobarbital (650 mg/kg Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). Blood was withdrawn by cardiac puncture using heparin as anticoagulant and centrifuged (20 minutes, 8 000 g, 4°C). Plasma samples were stored at –20°C until use. Livers were removed, rinsed by cold saline, dissected out and weighed within 2–4 min. Liver samples for biochemical analysis were immediately frozen in liquid nitrogen and stored at –80°C until use.

Serum enzymes assays

The serum level of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) as marker of liver injury was assayed. ALT was measured by colorimetric method [30] Results were expressed as international units per liter (IU/L). LDH activity was measured as the rate of oxidation of NADH with pyruvate at 340 nm and 25°C and expressed in units per liter (U/l) [6].

Preparation of extracts and liver enzyme assays

Frozen liver samples were homogenized in ice-cold saline (1:10, w/v) using an Ultra-Turrax homogenizer. Homogenates were centrifuged at 4°C and 10,000 g for 10 min. Supernatant fractions (extracts) were used for enzymes assay**.** The total supernatant protein was measured according to. Bradford [9] (Bio-Rad microplate assay kit). Tissue glutathione reductase (GR) activity was measured at 20 °C by the method of Mize and Langdon. [24]. Selenium-dependent glutathione peroxidase (GPx 1) and total glutathione peroxidase activities were measured in a GSH-reductase coupled assay with H_20_2 and H_20_2+tert-butyl hydroperoxide, respectively, as substrates according to Flohe and Guenzler [11]. Selenium-independent GPx activity (GPx 2) was calculated as the difference between total and selenium-dependent GPx. Tissue glutathione S-transferases (GST) activity was measured by the method of Habig et al. [13] with chloro-2,3-dinitrobenzene (CDNB) as the substrate. Total superoxide dismutase (SOD) activity was measured by indirect spectrophotometric methods based on the inhibition of the auto-oxidation of quercetin as described previously [19].

Preparation of extracts and GSH/GSSG assays

Frozen liver samples were homogenized (1:10 w:v) using a Ultra-Turrax homogenizer in ice-cold 2.5 % perchloric acid. Homogenates were centrifuged at 4°C and 10,000 g for 5 min in an Eppendorf centrifuge. Supernatants were removed and used for the measurement of total glutathione (total GSH = GSH + 2 GSSG) according to the enzymatic cycling method [33]. Total GSH was quantified in reaction media (400 µl) containing 40 µl sample, 0.2 U/ml GR, 0.2 M Tris (pH 8.0), 0.15 mM NADPH, and 0.5 mM DTNB by following the rate of reduction of DTNB by GSH at 412 nm and comparing this to a GSH standard curve. Reduced GSH was assayed as total GSH with the exception that GR was omitted. In order to quantify only GSSG, the method of Griffith [12] was used with slight modifications.

Metallothionein (MT) measurement by Cadmium-Hemoglobin Assay

Hepatic MT levels were determined by a cadmium-hemoglobin (Cd-heme) assay as described previously [27]. The method used radioactive ^{109}Cd, and the concentration of MT was calculated in each sample by measurement of ^{109}Cd radioactivity with a gamma counter (1272 Clinigamma, LKB Wallac; Turku, Finland). This was converted to MT concentration on the basis of 7 g-atoms of Cd per molecule of MT. The total hepatic MT concentrations were expressed as micrograms per gram of wet tissue.

RT-PCR

Total RNA was isolated from frozen liver samples using GenElute Total RNA miniprep kit (Sigma) and reverse transcribed (2 µg) using Superscript RT II (Invitrogen) to generate cDNA in a final reaction volume of 20 µl. Real-time quantitative PCR was performed using a LightCycler II Roche Diagnostics GmbH, (Germany) with SYBR Green JumpStart kit (Sigma). PCR conditions used for all reactions were 94°C, 5 s/55°C, 24 s/72°C, 1 s/80°C for 35 cycles. Amplification was performed with 2.0 mM $MgCl_2$, 0.2 mM dNTP, 2.0 U *Taq* polymerase, and 2 µM 5' and 3' oligonucleotide primers. Primers were selected from published sequences: mouse glutathione reductase [32]; mouse copper-zinc SOD [7]; mouse p-1 and p-2 GSTs p-1 and p-2 [5]. The sequences of the 5' and 3' primer pairs used were the following: sense GCGGCGCGCGCGCTCACC and antisense GCGCCGAGCTCCGCCGCCCGC for GR; sense GTCGGCTTCTCGTCTTGCTC and antisense CTTTCTTCATTTCCACCTTTGC for CuZnSOD, sense

ATGAGAATGGTAAGAATGAC and antisense TTATTAGTGCTGGGAAAACGG for GSTs p-1 and p-2. The amount of specific mRNA in samples were calculated from the standard curve, and normalized with the GAPDH rRNA (primers: sense ACCACAGTCCATGCCATCAC and antisense TCCACCACCCTGTTGCTGTA).

RESULTS AND DISCUSSION

Antiradical activity of flavonoid metal complexes

The scavenger efficacy of rutin and (–)-epicatechin metal complexes towards superoxide was quantitatively evaluated using illumination of riboflavin in the presence of tetramethylethylenediamine. Upon reoxidation on air photochemically reduced flavin generated superoxide anions, which in their turn reduced NBT. The reaction was nearly completely inhibited by SOD at the concentration of 1.5 nM (I_{50}=0.15 nM). Flavonoids and their metal complexes also effectively inhibited NBT reduction and the degree of inhibition is increased in a concentration-depended manner for all compounds studied that allowed calculating I_{50} values by regression analysis (Table 1). The I_{50} values clearly indicate that the scavenger potencies of flavonoid metal complexes are manifold more than those of the parent flavonoids. This finding supports that metal ions in complex with flavonoids may act as radical scavenging center in accord with the following reactions:

$$Me^{n+} + O_2^{\bullet -} + 2H^+ \rightarrow Me^{(n+1)+} + H_2O_2$$

Since the superoxide scavenging properties of flavonoid-ferric complexes were identical to those of flavonoid-ferrous complexes the scavenging mechanism through reduction of metal ions by superoxide may be also suggested:

$$Me^{(n+1)+} + O_2^{\bullet -} \rightarrow Me^{n+} + O_2$$

A crucial point related to potential medical application of metal complexes including flavonoid metal complexes is stability of the compounds in the presence of low molecular mass chelators and proteins. Since albumin is a predominant protein in serum and possesses strong ability to bind various metal ions effect of albumin on stability of flavonoid-metal complexes was studied. In these experiments ability of iron (II) and copper (II) complexes of rutin and (–)-epicatechin to inhibit superoxide-driven reduction of NBT was assayed before and after pretreatment (up to 60 min) with albumin. As can be seen from the data summarized in Table 2, the most stable was complex of rutin with iron (II) whereas metal complexes of epicatechin were relatively unstable. Significant affect of albumin on the antiradical properties of these complexes was found at a concentration of only 0.2 mg/ml.

It is widely accepted that toxicity of iron and copper is mainly related their ability to generate extremely active hydroxyl radical ($^{\bullet}OH$) in the presence of hydrogen peroxide (Fenton reaction). Chelation of metal ions by low molecular mass chelators results in strong influence on Fenton chemistry. Metal ions bound to certain chelators (e.g. desferrioxamine) may be less effective or even ineffective as Fenton's catalyst. Such chelators are often discussed as effective antioxidant. The ability of flavonoid-metal complexes to catalyze homolytic cleavage of hydrogen peroxide was studied using spectrophotometric approach. In these experiments various concentration of hydrogen peroxide were added to the solution with rutin-iron (II) or rutin-copper (II) complexes. Metal ions was supposed to act as Fenton catalyst while ligand moiety was a target for hydroxyl radical:

$$H_2O_2 + Me^{n+}\text{-}[Fl] \rightarrow {}^{\bullet}OH + {}^{-}OH + Me^{(n+1)}\text{-}[Fl]$$

$$^{\bullet}OH + Me^{(n+1)}\text{-}[Fl] \rightarrow 2^{-}OH + Me^{(n+1)}\text{-}[Fl_{ox}]$$

The rates of $^{\bullet}OH$ dependent rutin oxidation was calculated on the basis of the differential coefficient of extinction between flavonoid reduced and oxidized forms = $7.2 \cdot 10^3 \cdot M^{-1}cm^{-1}$ [31]. Rutin-iron complex was found to be relatively poor Fenton catalyst and marked ligand oxidation was found only under a large excess of hydrogen peroxide (Table 3). At the same time rutin-copper complex was substantially more effective in hydrogen peroxide decomposition and the rate of ligand oxidation was one order higher in comparison with that of rutin-iron(II) complex. In the following experiments a hydroxyl radical trap mannite (mannitol) was used in order to inhibit $^{\bullet}OH$ dependent ligand oxidation. However, there was no effect of mannite on the oxidation of rutin bound with iron or copper even under a large excess (500 times) of mannite (Table 3). This finding indicates that ligand oxidation resulting from the decomposition of hydrogen peroxide by rutin-

Table 1. Concentrations of flavonoid metal complexes (1:1) and free ligands inhibiting the superoxide-driven reduction of NBT by 50%

Flavonoids	I_{50} Values (μM)			
	Free ligands	Complexes with metals		
		Cu^{2+}	Fe^{2+}	Fe^{3+}
Rutin	9.0	0.5	2.7	2.5
(–)-Epicatechin	1.3	0.3	0.3	0.3

Table 2. Effect of pretreatment rutin and (–)-epicatechin metal complexes with albumin on the their ability to inhibit (percent inhibition) superoxide-driven reduction of NBT

Conditions	Without albumin	Preincubation with albumin, min		
		5	20	60
Rutin-Fe^{2+}, 8 μM	82 ± 7			
+albumin 0.5 mg/ml		82 ± 6	80 ± 7	76 ± 7
+albumin 2.0 mg/ml		73± 11	62 ± 8	57 ±.7
Rutin-Cu^{2+}, 4 μM	83 ± 6			
+albumin 0.2 mg/ml		82 ± 1	82 ± 1	83 ± 2
+albumin 0.5 mg/ml		60 ± 6	38 ± 0.7	31 ± 5
Epicat-Fe^{2+}, 1 μM	65 ± 3			
+albumin 0.2 mg/ml		63 ± 3	54 ± 3	44 ± 1
Epicat-Cu^{2+}, 1 μM	70.0 ± 5			
+albumin 0.2 mg/ml		49 ± 1	45 ± 3	40 ± 1

*Data are mean ± SD of five experiments.

Table 3. The rate of ligand (rutin) oxidation (*v*, μmol/min) as a result of hydrogen peroxide (200 mM) decomposition by chelated Cu^{2+} and Fe^{2+} ions in PBS (pH 7.4) without and with various concentration of mannite.

Mannite, μM	Rut- Cu II*	Rut- Fe II*
0 (control)	1.8 ± 0.1	0.20± 0.01
100	1.9 ± 0.1	0.19 ± 0.02
1 000	1.9 ± 0.1	0.17 ± 0.03
5 000	2.0 ± 0.1	0.18 ± 0.03

*-initial concentration of flavonoid-metal complexes (1:1) 10 μmol/l.

metal complex may be site specific and $^{\bullet}OH$ produced under these conditions practically does not escape to the complex surrounding. Thus rutin-metal complexes may not only scavenge superoxide anions but also can safely decompose hydrogen peroxide.

Protective effect of rutin-iron (II) complex against acute oxidative stress and liver injury in MT-I/MT-II null transgenic mice induced by thioacetamide

In these experiments potential antioxidant properties of flavonoids-metal complexes were investigated in vivo. Rutin-iron (II) complex was selected as effective and the most stable antiradical agent. In this study, we had to find a suitable model to test the pathology associated with oxidative stress and overproduction of ROS. Acute hepatic injury in mice induced by injections of thioacetamide was evaluated for this purpose. Thioacetamide (TAA) is a thionosulfur-containing compound and can cause severe hepatotoxicity by oxidative stress. The overproduction of reactive oxygen species (ROS) and oxidative modification of cellular constituents with TAA administration are due to its metabolic activation by cytochromes P-450-containing monooxygenases in endoplasmic reticulum of hepatocytes [29], activation of Kupffer cells [3], and down-regulation of dismutase, catalase and glutathione peroxidase which are responsible for elimination of superoxide anion and the breakdown of hydrogen peroxide [22]. Involvement of ROS in TAA hepatotoxicity was supported by studies demonstrating the protective effects of antioxidants such as thioredoxin and melatonin [10,26]. In a preliminary study, it was found that pretreatment of WT mice with flavonoid-metal complexes resulted in twofold increase of the liver level of metallothioneins (MT) at 12 h after TAA administration in comparison with mice treated with TAA alone. Since MT is well known endogenous antioxidants [4,14,23] a possible protection against oxidative injury in WT mice can result from increasing the liver MT levels rather than antioxidant activity of iron complex. No increase of low basal level of MT was found in MT-II null mice. Therefore, in order to exclude indirect protection through increasing liver metallothioneins, MT-I/MT-II null (knockout) mice were used for testing antioxidant properties of rutin-iron complex *in vivo*.

Table 4. Effects of 20 min pretreatment with rutin-iron complex on the acute liver injury assessed by plasma ALT and LDH 12 h after thioacetamide administration.

Treatment	ALT, U/l	LDH, U/l
Control	66 ± 16	1077 ± 935
TAA, 125 mg/kg	432 ± 334[a]	4495 ± 3666[a]
TAA + Rut-Fe, 7 mg/kg	109 ± 83[b]	1048 ± 1098[b]

*Data are mean ± SD of five animals.
ALT, alanine aminotransferase; LDH, lactate dehydrogenase, all expressed as units per liter at 22°C.
[a] - Significantly different (p<0.05) versus control; [b] - significantly different (p<0.05) versus TAA treatment by t-test.

Table 5. Effects of 20 min pretreatment with rutin-iron complex on the glutathione level (μmol/g tissue) and GSH/GSSG ratio in liver of MT-KO mice12 h after of thioacetamide treatment.

Treatment	Total GSH, μmol/g	GSH, μmol/g	GSSG, μmol/g	GSH/GSSG
Control	8.92 ± 1.23	7.72 ± 1.67	0.319 ± 0.094	25.8 ± 9.9
TAA, 125 mg/kg	4.56 ± 2.16[a]	2.93 ± 2.44[a]	0.366 ± 0.072	11.6 ± 4.0
TAA + Rut-Fe, 7 mg/kg	6.22 ± 1.70[b]	4.46 ± 1.75	0.320 ± 0.153	15.6 ± 4.4[b]

*Data are mean ± SD of five animals.
[a] - Significantly different (p<0.001) versus control; [b] - significantly different (p<0.05) versus TAA treatment by t-test.

It was found that pretreatment with rutin-iron complex protected against acute liver injury induced by intraperitoneal injections of thioacetamide (125 mg/kg body weight) as indicated by significantly decreased plasma ALT and LDL levels at 12 h following exposure (Table 4). Rutin-iron complex partially affected

the depletion of total and reduced glutathione and improved the GSH/GSSG ratio in liver of knockout mice at 12 h after intraperitoneal injections of thioacetamide (Table 5). The pretreatment with rutin-iron complex also prevented the loss in activity of selenium-independent glutathione peroxidase (GPx 2) and glutathione reductase and this effect was statistically significant (Fig. 1). However, the pretreatment with flavonoid-metal complex had no statistically significant effect on activities of selenium-dependent glutathione peroxidases (GPx 1), glutathione S-transferase and SOD in liver of both MT-KO and WT mice treated with TAA (data not presented).

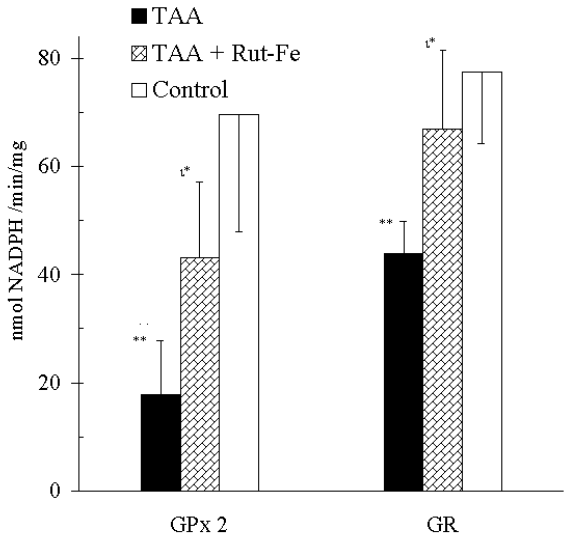

Figure 1. Effects of 20 min pretreatment with rutin-iron complex on activities glutathione peroxidase II and glutathione reductase in liver of MT-KO mice 12 h after of thioacetamide treatment. Data are mean ±SD of five animals.
Statistical significance: ** indicate $p<0.005$ versus saline treatment (control) at 12 h; ι* indicate $p<0.05$ versus TAA treated mice by T test.

The loss of the hepatic activities of antioxidant and glutathione utilizing enzymes after administration of TTA may be due to both: enzymes inactivation and impairment of protein synthesis as a result of negative effect of TTA-induced oxidative on expression of corresponding genes. To clarify possible effects of TAA administration on gene expression, mRNA levels of the GR, SOD and GST genes were examined by quantitative RT-PCR in liver of MT-KO mice at 12 h after intraperitoneal injections of thioacetamide with and without 20 min pretreatment with rutin-iron complex. GAPDH mRNA levels were also determined as internal control. Data were corrected with GAPDH levels and were expressed as fold of control knockout mice (Fig. 2). No significant differences were found in liver GR and SOD mRNA levels between all tree groups of mice, however significant (more than 60 %) increase of GST mRNA was found in liver of TAA treated mice as compared with control mice. It have be mentioned that pretreatment of MT-KO mice with rutin-iron complex resulted in prevention of the increase of GST mRNA. Therefore, effect of TAA on gene expression cannot lead to the loss of hepatic antioxidant and glutathione utilizing enzymes and their inactivation as a result of TAA-induced oxidative stress may be the main reason for this phenomena.

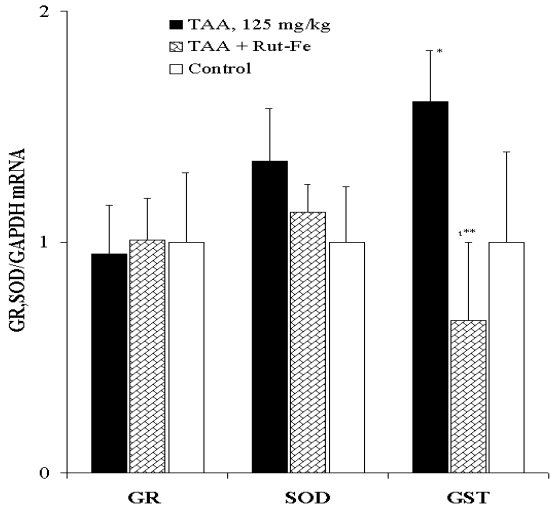

Figure 2. Effects of TAA on GR, GSTs and SOD mRNA levels in mice liver with and without 20 min pretreatment with rutin-iron complex. GR, SOD and GSTs mRNA levels were examined by RT-PCR. GAPDH mRNA levels were also determined as internal control. Data were corrected with GAPDH levels and were expressed as fold of control mice. Data shown are the mean ± SD
★, $p < 0.05$, compared with the control; ι** indicate $p<0.01$ versus TAA treated mice by t-test.

Thus, *in vivo* data confirm the crucial role of oxidative stress in TAA-induced hepatotoxicity and reveal protective effect of rutin-iron complex under these conditions. This effect may be due to both: direct free radical scavenger activity and indirect mechanism through the protection of key

antioxidant enzymes. Finally the results of this study suggest therapeutic potential of flavonoid-metal complexes for the treatment of oxidative stress-related diseases and dysfunction.

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