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ALTERATION OF GENES EXPRESSION OF COX1, TP53 AND FRRSI IN IRON OVERLOADED RABBITS: ROLE OF QUERCETIN AND DEFEROXAMINE (PART-III)

[1]MuntasserAlawi Awad and [2] Baraa Najim Al-Okaily

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Abstract

The present study aimed to investigate the role of quercetin (a naturally occurring chelating agent) and deferoxamine in the gene expression of Cox-1 and TP53 of the heart and FRRSI of the duodenum in iron-overloaded rabbits. To examine whether quercetin and deferoxamine mitigate the change in the level of Cox-1 and TP53 expression in response to iron overload in rabbits, therefore. Twenty-eight rabbits (each group of seven) were used in the current study and treated for 28 days as follows: Group C: Animals in this group were intraperitoneally (IP) injected with normal saline every 72 hours, plus normal saline orally every day as a negative control. In the T1, T2, and T3 groups, the animals in these groups were IP injected with iron dextran (100 mg/kg) every 72 hours. Additionally, the animals in the T2 group were treated with quercetin (350 mg/kg) orally every day, and the animals in the T3 group were injected with deferoxamine (DFO) (125 mg/kg) IM every day. After the end of the experiment, the rabbits were sacrificed, and specimens from the heart and duodenum were obtained to estimate the gene expression of COX-1, TP53, and FRRSI, respectively, using quantitative polymerase chain reaction (qRT-PCR). The results showed a significant increase in fold change in gene expression levels of COX-1 and TP53 in iron overload rabbits (group T1) compared with another group. Also, a significant difference in the level of COX-1 between T2 and T3, while a non-significant difference in the level of TP53 between T2 and T3 was observed. In contrast, the results showed a significant decrease in the level of FRRSI gene expression in group T1 as compared to the other experimental groups, while a non-significant difference was noticed between T2 and T3 for the same gene. Conclusion: The current study suggests using quercetin as an iron chelator is more effective in iron-overloaded rabbits by alleviating the changes in gene expression of COX-1, TP53, and FRRSI in the heart and duodenum.

Keywords: Flavonoids, Iron chelators, Iron overload diseases, Iron related genes.

Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad.

[1]Corresponding author: montasser.alawi1106h@covm.uobaghdad.edu.iq, muntasseralawadi0@gmail.com ORCID:0000-0001-9824-0755

[2]baraa.n.@covm.uobaghdad.edu.iq, ORCID:0000-0002-2691-2089

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Introduction:

The body needs iron to support physiological processes including DNA synthesis, oxygen and electron transport, and enzyme reactions (Fang et al., 2023). When storge proteins become saturated with iron, free iron accumulates in the tissue and plasma, this includes labile plasma iron and non-transferable bound iron, also cells take free iron to form labile iron pools (Hsu et al, 2022). iron overload produces reactive oxygen species (ROS) and contributes to oxidative stress (OS) (Hasan and Mahmoud, 2018; Hamed and Al-Qayim, 2019). It is well established that OS induces cardiovascular diseases as well as in iron overload (Júnior *et al*, 2017). Since iron overload can cause the dysfunction of many organs (Abood and Al-Okialy, 2019; Al-Rubaie et al., 2020).

Iron chelation treatment is a life-saving for disorders caused by an iron overload (AbdElkader and Aly, 2015; Reddy et al., 2022). Due to its extensive clinical history and inexpensive cost, the iron chelator deferoxamine (DFO) remains a first-line treatment choice for reducing iron load in patients with transfusion dependent thalassemia (Musallam et al., 2013). DFO binds with free iron in the plasma within cells at a 1:1 ratio (Tantiworawit *et al*., 2022). The iron that is bound with DFO and excreted through the urine or bile can be removed from myocardial cells by directly binding with it, DFO does not bind with iron that is bound with transferrin, hemoglobin, or cytochrome molecules, therefore, only a little amount of chelating iron is needed at any given time (Shawkat et al., 2018).

Flavonoids may have high antioxidant acti vities and be less toxic due to their unique structures. (Sakamuri et al., 2023; Khudiar and Naji, 2012). Therefore, it is widely used in medicine as a powerful antioxidant (Khalil et al., 2013).

Quercetin is an example of naturally flavonoids, it is found in different fruits and vegetables (AL-Awady and AL-Zamely, 2016). Quercetin, considered a potent antioxidant, and can act as chelating metals, scavenging ROS, and protecting against LPO (Awadi et al., 2016; HK et al., 2019). The mechanism by which quercetin treated the iron overload depends on its antioxidant properties and iron chelation (Chen et al., 2020). Many gene related with oxidative stress that induced by iron overload like Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase is an enzyme that is involved in the production of prostanoids such as thromboxane and prostaglandin (Litalien and Beaulieu, 2011). Activation of COX enzymes increase the level of prostaglandin F2α (PGF2α), also known as amoglandin, which causes inflammation and pain (Suzuki et al., 2015). Moreover, tumor suppressor genes, such as TP53, have a function in iron homeostasis, which is controlled by several key regulators of iron metabolism, TP53 appears to affect the expression of genes involved in both systemic and cellular iron homeostasis (Zhang and Chen, 2019). Besides, FRRS1 gene for ferric reductase enzyme, play important role in iron absorption which catalyzes the reduction of Fe^{3+} to Fe^{2+}, before the iron can be taken up in the enterocytes (Milman, 2020).

So, this work aimed to investigate the effect of quercetin or DFO on some genes expression of heart and duodenum related to iron overload in rabbits.

Materials and methods:

Experimental design:

Twenty-eight adult male rabbits were used in the current study; six months old and weighed between (1.7 - 2 kg). They were obtained from the animal's house at the Veterinary Medicine College/University of Baghdad. The animals were reared under controlled conditions at 22°–25°C. Animals were divided randomly into four groups (each of

seven) and treated for 28 days as follows: Group C: Animals were intraperitoneally IP with normal saline every 72 hours + normal saline orally each day (negative control), while T1, T2, and T3 groups were I/P injected with iron dextran (100 mg/kg) every 72 hours (Khudiar and Naji, 2012). Furthermore, rabbits in the T2 group, were treated with quercetin (350 mg/kg) orally daily, and in the T3 group, were injected with DFO (125 mg/kg) IM daily (El-Sheikh et al., 2018).

Tissue Samples Collection:

Through the experiment, the animals were weighed weekly because quercetin and DFO were given according to weight. Ethical issues have been checked by the authors and at the end of experiment, animals were sacrificed by overdose of Ketamine + xylazine, then tissue specimens were collected from the heart and duodenum and put in Trizol to estimate gene expression of COX 1, TP53, and FRRS1 genes (Pahlevan, 2014).

Table (1) illustrate Primers Used in Present Study with Their References:

Primer	Sequence (5'-3')		Product Size	NCBI Reference
Mitochondrial Cytochrome C Oxidase Cox Gene	**F**	**ACCAAGAGGATGCTGGACATG**	**71bp**	**NM_001361489.1**
	R	**TTCTTGTCGTAGTCCCACTTGG**		
Ferric Chelate Reductase FRRS1 Gene	**F**	**AAGCAGTGCCCCAAATCATG**	**109bp**	**XM_002715840.3**
	R	**TGGAAGTGCATTTGGTTGGG**		
Tumor Protein TP53 Gene	**F**	**TCAGGAGACGTTTTCAGACCTG**		
	R	**TTGAGCCAGTTTGCAACGTC**	**117bp**	**NM_001082404.1**
Glyceraldehyde-3-Phosphate Dehydrogenase GAPDH Gene	**F**	**ATGCCCCCATGTTTGTGATG**	**75bp**	**NM_001082253.1**
	R	**AGGATGCGTTGCTGACAATC**		

Screening of genes:

Total RNA extraction:

Total RNA was extracted from heart and duodenal specimens by using TRIzol® reagent kit (Bioneer/ Korea) and done according to company's instructions

Total RNA yield estimation:

RNA that was extracted was assessed to determine the RNA quantity (ng/µL), and RNA purity by using a nanodrop spectrophotometer (THERMO. USA), at absorbance at 260 and 280 nm.

cDNase I Treatment:

DNase I enzyme kit (Promega /USA) was used to remove DNA from extracted RNA and done according to company's instructions

cDNA synthesis:

Treated RNA with the DNase-I enzyme used to synthesize cDNA by using M-MV. Reverse Transcriptase Kit (Bioneer /Korea) and done according to company's instructions

Quantitative Real-Time PCR (qPCR):

Target genes expression was quantified by qPCR for (LOC100339628 gene for mitochondrial cytochrome c in cardiac muscle, the TTP53 gene for TP53 in cardiac muscle, and the FRRS1 gene in the intestine), housekeeping (GAPDH,) gene was used to normalize control and experimental samples by using Real-Time PCR technique.

qRT-PCR data analysis:

The results of qRT-PCR for target and housekeeping genes were analyzed by the relative quantification gene expression levels (fold change) (the ΔCT Method Using a Reference Gene) that described by (Livak and Schmittgen, 2001).

Statistical analysis:

The data was analyzed statistically using the computer program SPSS version 24. The values expressed as mean± SE. Statistical analysis performed basis on one way ANOVA with lest significant difference (LSD) at P0.05 was used to compare between groups (Baarda *et al*., 2019).

Result:

The results in table (2) and Figure (1) that expressed as fold change of gene expression level of COX1 gene revealed a significant ($p \leq 0.05$) increase in the T1 group (7.78±0.54) compared with other groups. Also, a significant decrease of this gene expression compared with T2 and T3 groups (3.91±0.24), (3.47±0.43) respectively compared with the T1 group, while T2 group (3.91±0.24) showed a significant increase compared with T3 group (3.47±0.43).

At the same table and Figure (2) that expressed as fold change of gene expression level of TP53 gene revealed a significant ($p \leq 0.05$) increasing in the T1 group (6.04±0.19) compared with other groups. C group (1.01±0.07) showed a significant decrease in compared with T2 and T3 groups (3.41±0.29), (3.04±0.03) respectively, while T2 group and T3group not showed any significant difference between them.

The fold change of gene expression level revealed a significant ($p \leq 0.05$) increasing in the T2 and T3 groups (1.56±0.17), (1.46±0.20) respectively compared with other groups, table (2) and Figure (3), while the T1group (0.19±0.01) showed a significant decrease compared with control group (0.79±0.02).

Table (2) Effect of Quercetin or DFO on COX1, TP53 and FRRS1 genes of Adult Rabbits Exposed to Iron Overload by Iron Dextran for 28 days.

Groups / Parameters	C	T1	T2	T3
Cox1	d 1.01±0.08	a 7.78±0.54	b 3.91±0.24	c 3.47±0.43
TP53	c 1.01±0.07	a 6.04±0.19	b 3.41±0.29	b 3.04±0.03
FRRS1	b 0.79±0.02	c 0.19±0.01	a 1.56±0.17	a 1.46±0.20

Values are expressed as mean± SE. n= 7.

Group C: Animals were injected i/p with normal saline every 72 hours + normal saline orally each day (negative control). T1, T2, and T3 groups were injected i/p with iron dextran (100 mg/kg) every 72 hours. Additionally, T2 group animals were treated with quercetin (350 mg/kg) orally daily, and T3 group animals were injected with DFO I/M (125 mg/kg) daily.

Different letters= Significant Differences ($p<0.05$). between groups

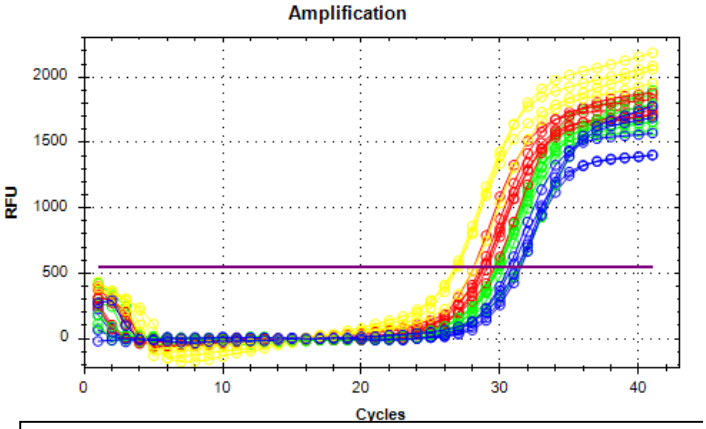

Figure (1) The Real Time Amplification Plots of Cox1 Gene expression of heart tissue in Rabbit Experimental Samples. Where, The Blue Plots (Control Group), The Yellow Plots (T1 Group), The Red Plots (T2 Group), And the Green Plots (T3 Group).

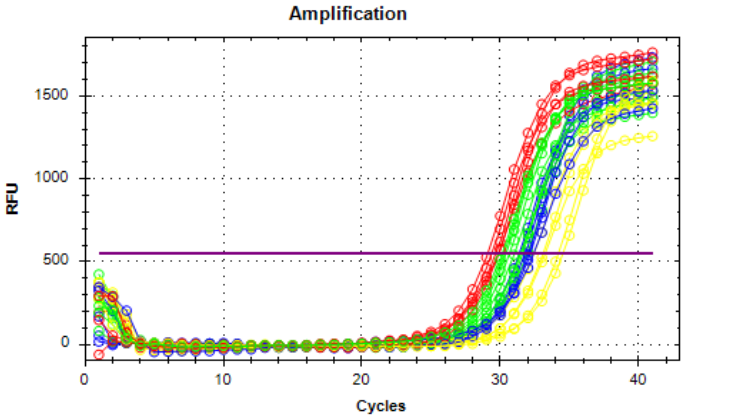

Figure (3) The Real Time Amplification Plots of FRRS1 Gene expression of heart tissue in Rabbit Experimental Samples. Where, The Blue Plots (Control Group), The Yellow Plots (T1 Group), The Red Plots (T2 Group), And the Green Plots (T3 Group

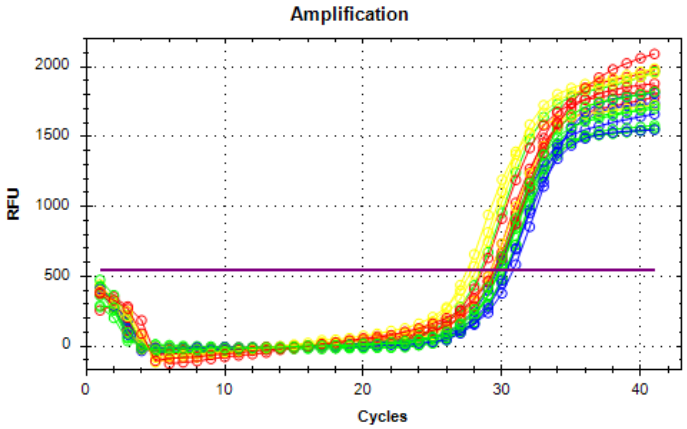

Figure (2) The Real Time Amplification Plots of TP53 Gene expression of heart tissue in Rabbit Experimental Samples. Where, The Blue Plots (Control Group), The Yellow Plots (T1 Group), The Red Plots (T2 Group), And the Green Plots (T3 Group).

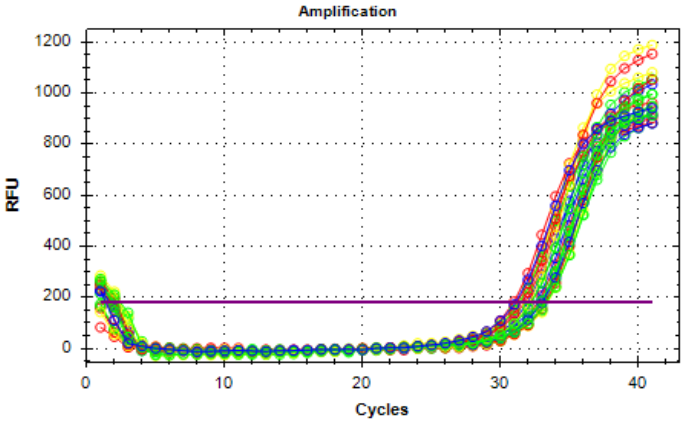

Figure (4) The Real Time Amplification Plots of Housekeeping GAPDH Gene in Rabbit Experimental Samples. Where, The Blue Plots (Control Group), The Yellow Plots (T1 Group), The Red Plots (T2 Group), And the Green Plots (T3 Group).

Discussion:

Current results based on the mRNA heart tissue showed over expression of COX1 gene linked to iron overload in the T1 group, and that this plays a significant role in progressive of myocardial dysfunction, these results agreement with (Kim et al., 2001; Shi and Vanhoutte, 2008; and Wei et al., 2022). COX, also known as prostaglandin-endoperoxide synthase is an enzyme that is involved in the production of prostanoids such as thromboxane and prostaglandin. (Litalien and Beaulieu, 2011). COX enzymes activation increases prostaglandin F2α level, which causes pain and inflammation, the COX inhibitory test is an estimation of PGF2α production directly from prostaglandin H2 (Suzuki et al., 2015; Li et al., 2022). Iron overload related to the OS in a human carcinoma cells line upregulate the COX1 expression that trigger inflammatory response (Handa et al., 2019). Pathways associated with ROS detoxification and TNF production were enhanced in severe alcoholic hepatitis patients with iron overload, suggesting that iron accumulation increases inflammation primarily through TNF-signaling activation. (Yas, 2020). The results suggests that quercetin suppresses the early stages of inflammation through other proinflammatory mediators such as COX1. Our results agreement with (Ohta et al. 2006; Lesjak et al., 2018). Previous study was found that quercetin can inhibit gene expression also, it can be activated of peroxisome proliferator activated receptor C, leading to the prevention of inflammation (Li *et al*., 2016). Quercetin has ability to expressed COX1 gene and consequently suppresses the inflammation by reducing the COX1 gene expression. Despite the exact mechanism of inhibit COX activity by flavonoids is unknown (Catarino et al., 2016). However, supposed the mechanisms reactions of COX-1 include hydrogen abstraction from arachidonic acid leads to the free radical formation, where quercetin could serve as free radical scavengers (Lesjak et al., 2018; Deng et al., 2021).

The results showed that DFO inhibited COX1 activity compared to T1 and T2 treated groups maybe by interaction directly with the POX active site of enzyme, DFO effect on COX1 is in line with previous study (Sun et al., 2020; Zhu et al, 2023). DFO, its suppressed inflammation and ferroptosis via iron chelator mechanism and down-regulate the expression of COX1 that trigger inflammatory responses (Yu et al., 2021). Various researches revealed that DFO decreased ferroptosis and cytosolic ROS production, as well as that DFO also has a cardioprotective effects via inhibiting inflammation, cytosolic ROS production and ferroptosis (Ooko et al., 2015; Van Opdenbosch and Lamkanfi, 2019).

Our results based on the mRNA-heart tissue, clearly show that genes linked to tumor suppressor, including expression of TP53, a significantly expressed of TP53 in iron overload treated rabbits compared to other experimental groups current result in constancy with (Zhang et al., 2017). Recent study found that TP53 play a role in iron homeostasis also, regulated by numerous modulate the genes expression that involving in cellular and systemic iron homeostasis (Zhang and Chen, 2019) TP53 is able of increasing ferritin expression, while lowering TFR1 expression by a posttranscriptional mechanism (Zhang et al., 2008). Additionally, it was found that the iron-sulfur cluster assembly enzyme (ISCU) is a TP53 target, and the ISCU protects the cells from iron overload; therefore, ISCU serves as a TP53 mediator to maintain the cellular iron pool (Funauchi et al., 2015). In this field, ferritin was found to activate expression of TP53 under OS via ferritin binding to TP53 then increases transcription activity of the TP53 which is ferritin independent ferroxidase activity also, ferritin reduces the TP53 target genes induction in response to hydrogen peroxide therapy (Shi et al., 2015).

Current finding showed the inhibitory effects of quercetin on the TP53 expression in group T2 compared with iron group T1, this agreed with Soleimani and Sajedi, (2020) who found the quercetin prevented the synthesis and accumulation of newly TP53 protein (Wang et al., 2021). Thus, our results summarized that dietary quercetin could downregulate TP53 expression more efficiently in iron overload rabbits, providing new information about the mechanism of its action. Whereas, the group treated with quercetin showed increased regulation of the TP53 gene that agreed with Chan et al. (2013), who found quercetin increases the antitumor activates through upregulation of TP53 protein expression. The inability of quercetin to reduce the gene expression of TP53 nearly to that of control group may by in a dose and time depended manner in the current study thus, precise mechanism by which quercetin enhanced expression of TP53 in the current study remain unclear.

The inhibitory effects of DFO on TP53 gene expression in T3 group compared with iron group (T1) and enhanced TP53 expression compared with control group was observed, this disagreed with Watanabe and Kaetsu, (1995), who found DFO caused iron deprivation and increase expression of TP53 in ML-1 and Raji cells. And agreement with (Huang et al., 2000). Iron chelation medications enhances mitochondrial dysfunction and restores TP53 signaling in leukemic cells by stabilizing TP53 family members. (Calabrese et al., 2020). Several studies that found, the oral iron chelator delays leukemic transformation in Myelodysplastic Syndromes (MDS)patients (Chen et al., 2017; Hoeks et al., 2020Hydroxyl radical is responsible for activation of TP53, DFO which chelated iron to make it unable to generating OH radical from H_2O_2, consequently leads to diminished TP53 activation (Wang et al., 2000).

Our results based on the mRNA-duodenum tissue, clearly show down regulation of the FRRS1 gene, in the apical membrane of enterocyte of iron overload animals, as a result of iron abundance in the body and the not need to absorb additional amounts of iron from intestine, this agreed with (Milman, 2021). Its increase during iron deficiency and decrease during iron overload and repletion (Steinbicker and Muckenthaler, 2013). Under conditions of iron deficiency, hypoxia inducible factor 2 alpha stabilization leads to ferric reductase upregulation in the apical membrane of enterocytes and increased ferroportin expression on the basal membrane (Drakesmith et al., 2015).

It has been found an upregulation of FRRS1 gene in theT2 and T3 groups compared with the iron group, and several previous studies on the putative protective role of quercetin revealed a decrease in serum iron following quercetin treatment in iron overloaded rats (Lesjak et al., 2014; Xiao et al., 2023). There is little information about DFO and quercetin effects on the intestinal mucosa with the iron overload however, in the current study, quercetin efficiently mor than DFO in iron chelation from the intestine and circulatory pools. Quercetin reduced iron absorption in the intestine by chelating iron with its 3-hydroxyl group or by regulating ferroportin transporter expression, and it increased iron excretion in iron overload (Cabantchik et al., 2005; Lesjak et al., 2014). In the current experiment the T2 and T3 groups treated with both quercetin and DFO separately showed up-regulation of FRRS1 gene expression in mucosal tissues of duodenum in both groups, there have been no studies about the effect of quercetin or DFO on the FRRS1gene, however, a pervious study found that DFO and quercetin caused a rapid reduction in small intestine iron (El-Sheikh et al., 2018).

Conclusion:

Quercetin and DFO lead to the downregulation of COX1 and TP53 gene expression in heart tissue and the upregulation of FRRS1 gene expression in duodenum tissue in iron overload rabbits, but the quercetin as an iron chelator is more effective in iron-overloaded rabbits.

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Conflict of Interest:

There is no conflict of interest.

Authors' Contribution:

The experiment was designed and the study was supported technically by Baraa Najim Al-Okaily. Muntasser Alawi Awad performed the experiment and analyzed and interpreted the data.

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