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Studies on the protective functions of natural compounds against iron-induced toxicity in cell and mouse models

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**Studies on the protective functions of natural compounds
against iron-induced toxicity in cell and mouse models**

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In fulfilment of the requirements for the degree of Doctor of
Philosophy (PhD) in Diabetes and Nutritional Sciences

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Abstract

Type 2 diabetes (T2D) is a chronic metabolic condition characterized by insulin resistance and pancreatic β -cell failure. Over the past decade, the incidence of T2D has been increasing, and is of growing concern, as T2D is often associated with obesity. Excess iron has been shown to induce ferroptotic pancreatic cell damage caused by oxidative stress due to increased production of reactive oxygen species (ROS). This facilitates the progressive impairment of β -cell function in the pathogenesis of T2D. The present thesis presents two novel therapeutic strategies for iron related damage in T2D management; the first approach sought to attenuate oxidative stress with different polyphenols and the second is an inducing antioxidant pathway by polyphenols against excess iron.

Studies were conducted to gather evidence on the protective functions of various polyphenols in both *in vitro* cell and *in vivo* mouse models. This research presents data on the protective effect of curcumin and (-)- epigallocatechin-3-gallate (EGCG), against iron loading and erastin-mediated ferroptosis in MIN6 cells. Curcumin and EGCG both significantly protected pancreatic cells against iron-induced oxidative damage and erastin-induced ferroptosis. The polyphenols enhanced cell viability in erastin-treated MIN6 cells in a dose- and time-dependent manner. Furthermore, MIN6 cells exposed to erastin alone showed elevated levels of iron, glutathione (GSH) depletion, glutathione peroxidase 4 (GPX4) degradation and lipid peroxidation compared to cells that were protected by co-treatment with curcumin or EGCG. Taken together, the data identify curcumin and EGCG as novel ferroptosis inhibitors, which might exert their protective effects by acting as iron chelators and preventing GSH depletion, GPX4 inactivation, and lipid peroxidation in MIN6 cells. The implications of the findings on the effects of iron overload and ferroptosis represent a potential therapeutic strategy against iron-related diseases.

Antioxidant therapy is a promising therapeutic strategy to prevent or even reverse the progression of iron-induced oxidative stress and ferroptosis by activating nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. Thus, phenolic acids, ferulic acid (FA) and, its metabolite Ferulic Acid 4-O-Sulfate Disodium Salt (FAS) were evaluated against oxidative stress and ferroptosis in both *in vitro* cell and *in vivo* mouse models. FA and FAS both significantly protected MIN6 and HepG2 cells against erastin-induced ferroptosis and iron related oxidative stress. Phenolic acids corrected oxidative stress markers in iron overloaded mice models. The prospects of those phenolic acids to reduce oxidative stress and ferroptotic cell damage will be of immense benefit in improving patient outcomes.

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Abbreviations

ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAT	Catalase
Dcytb	Duodenal cytochrome b
DMEM	Dulbecco's modified eagle medium
DFO	Deferoxamine
DFP	Deferiprone
DFX	Desferrioxamine
DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FA	Ferulic Acid
FAS	Ferulic Acid 4-O-Sulfate Disodium Salt
Fe (II)	Ferrous iron
Fe (III)	Ferric iron
FeCl ₃	Ferric chloride
FeSO ₄	Ferrous sulfate
FPN	Ferroportin
FtL	Ferritin light chain
FtH	Ferritin heavy chain
Ferrozine	Sodium 3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-triazine
FS	Fenton substrate
Foxo1	Forkhead box O1
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit
GPx	Glutathione peroxidase
GPx4	Glutathione peroxidase-4
GSH	Reduced glutathione
H ₂ O ₂	Hydrogen peroxide
Hb	Hemoglobin

HCl	Hydrochloric acid
HO-1	Haem-oxygenase-1
ICP MS	Inductively-coupled plasma mass spectrometry
ID	Iron Dextran
MDA	Malondialdehyde
MEM	Minimum essential medium eagle
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
Nrf2	Nuclear factor erythroid 2
NQO1	NAD(P)H Quinone Dehydrogenase 1
PBS	Phosphate-buffered saline
PDX	Pancreatic and Duodenal Homeobox 1
ROS	Reactive oxygen species
RPL19	Ribosomal protein L19
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SSA	Sulfosalicylic acid
TBST	Tris-buffered saline with tween
TfR1	Transferrin receptor-1
T2D	Type 2 diabetes

Chapter 1. General Introduction

1.1. Iron overloads, disorders, and metabolism

Iron is a potent pro-oxidant and has a finely-tuned control mechanism related with regulation of iron entry, iron recycling and loss of body iron (1). Iron can be retained in the body and there is a high risk of iron overload and iron-related toxicity. Iron accumulation in critical organs such as liver, brain, heart, and pancreas cause organ dysfunction due to the generation of oxidative stress. Some are known to be hereditary, while in others the genetic and hereditary basis has not been confirmed (2). A large percentage reflect systemic iron overloads, such as hereditary hemochromatosis (HH), post-transfusion siderosis, while others result in regional or local iron accumulation because of inflammatory or other disease processes, such as chronic liver diseases. Finally, an increasingly recognized class of iron disorders is because of disrupted intracellular (Friedreich ataxia) or body iron traffic (anaemia of chronic diseases) leading to iron misdistribution despite normal total body iron content (Table 1.1) (3).

Table 1.1. Hereditary disorders associated with iron overload and iron misdistribution (4)

Iron overload		Iron mis-distribution	
Disorder/cause	Pattern of iron accumulation	Disorder/cause	Pattern of iron accumulation
Hereditary Hemochromatosis	Systemic (iron accumulation in parenchymal cells)	X-linked sideroblastic anemias	Systemic (mitochondria)
Ferroportin Disease	Systemic (preferential iron accumulation in macrophages)	Friedreich ataxia	Systemic (mitochondria)
Aceruloplasminemia	Systemic	Neuroferritinopathy	Regional (brain)
Atransferrinemia	Systemic		
DMT-1 deficiency	Regional (mainly liver)		
H-ferritin-related iron overload	Systemic		
Hereditary iron overloading anemias with inefficient erythropoiesis	Systemic (early iron accumulation in hepatocytes due to increased iron absorption)		

1.2. Excess iron and T2D

Type 2 diabetes (T2D) is a chronic metabolic disorder of glucose metabolism, and one of the most prolific and serious public health challenges of the 21st century (4). The incidence rate has dramatically increased during the past three decades and it is projected to have an effect on more than 300 million people by 2025 all over the world (5). Research on T2D has focused on macronutrients, particularly carbohydrate and fat while a close link between abnormal glucose metabolism and iron, has also been noted in individuals with pathological iron overload, where the systematic iron amount in the body is elevated, in hereditary hemochromatosis, transfusional iron overload as in thalassemia and African iron overload (6).

Principally, iron generates ROS to control glucose oxidation and insulin secretion. However, it should be emphasized that a low level of ROS is essential for cell signalling molecules that mediate cellular differentiation, survival, and metabolism. Conversely, excess iron exhibits deleterious effects on cellular homeostasis in in vitro studies (7). Also, excess iron plays a deleterious role in pancreatic β cells through increasing ROS, resulting in impaired insulin synthesis and excretion (8). ROS have been hypothesized to contribute to the development of insulin resistance and β cells destruction, which might be associated with an increased risk of T2D (8,9). Pancreatic islets are highly vulnerable to iron overload-mediated oxidative stress, leading to impaired insulin signalling, mitochondrial dysfunction, inhibition of ATP production, and decreased insulin (7,8). Epidemiological studies have reported remarkable associations between excess iron stores and T2D, although some of these findings are not fully consistent (8,9). The reason for the discrepancy was the use of ferritin, an iron storage protein, and an acute-phase protein, as a biomarker to assess iron body stores. Consequently, ferritin is elevated in inflammation, cancer, liver disease, and some other conditions associated with diabetes such as obesity and metabolic syndrome (9). Moreover, ferritin is correlated positively with dyslipidaemia biomarkers and hepatic enzymes, and it is negatively associated with adiponectin. Thus, the effect of iron overload on pancreatic β cells and their function is not clearly defined in the literature (10). Iron overload affects several tissues involved in lipid and glucose metabolism such as the muscle, liver, pancreatic beta cells and adipose tissue, and organs influenced by chronic diabetic complications (11). Epidemiological studies have reported significant associations between body iron stores and chronic metabolic diseases, although some of these findings have not been fully consistent (11). Ferritin is elevated in

inflammation, cancer, liver disease and some other conditions associated with diabetes, such as obesity and metabolic syndrome. Moreover, ferritin is correlated with dyslipidaemia biomarkers, hepatic enzymes and negatively associated with adiponectin. However, studies that adjusted for such confounders showed statistically significant associations (11,12).

β thalassemia is a hereditary haemoglobin disorder, in which patients become iron overloaded because of several transfusions required to maintain adequate erythrocyte levels, suggesting that β thalassemia patients might have a high risk for T2D. A prospective cohort study including 748 β thalassemia patients with 11-years follow-up reported that almost 40% of patients developed T2D (13). Another prospective study revealed a prevalence of T2D three times that of sibling's control. Evidence suggests that iron plays a role in T2D even in the absence of pathological iron overload (14). A case control study of 27,548 participants in Germany showed that serum ferritin concentration significantly and inversely correlated with diabetes risk regardless of inflammation, insulin resistance, hepatic fat, and dyslipidaemia (15). Some other prospective cohort studies drew similar conclusions in different populations. For example, a prospective study among middle aged and elderly people in China, a country with the largest diabetic population worldwide, found that the risk of diabetes was increased by about twofold in subjects at the highest quantile of ferritin compared to the lowest after adjusting for body mass index, liver enzymes and adiponectin (8). The EPIC Norfolk cohort study also showed that serum ferritin was important as the predictive factor in the development of T2D in 360 clinically incident diabetes cases and 758 controls even after adjusting for other possible risk factors such as vitamin C level, interleukin 6, c-reactive protein, hepatic enzymes, fibrinogen, and adiponectin (16).

A causative link between iron overload and diabetes was suggested by improving T2D when iron stores are reduced (17). Experimental models showed a reduction in the incidence of T2D from 78 to 22% at 120 days of age after serial blood withdrawals in Belgrade (BB) rats (18). In humans, a study of 181 adult males showed that frequent blood donors had increased insulin sensitivity and less iron stores (19). Similar results were found in intervention studies that involved phlebotomy or iron chelation therapies (20).

Regarding insulin deficiency, pancreatic islets are extremely susceptible to oxidative stress possibly due to low expression of the antioxidant defence enzymes and the exclusive reliance on mitochondrial metabolism of glucose (21). Iron-induced oxidative stress can result in

pancreatic cell damage through insulin deficiency. In vivo, mice models of hemochromatosis (HH), characterized by excessive intestinal absorption of iron and accumulation of iron, showed increased expression of apoptotic genes (22). Death of β cells can consequently lead to diminished insulin secretion and therefore the development of T2D (23). Some cross-sectional studies have examined insulin secretion capacity in patients with HH. In one study, reduction in the insulin secretory capacity in subjects with HH was only confined to subjects with impaired glucose tolerance, whereas HH subjects with normal glucose tolerance had insulin secretion comparable to that of the controls (24). Another study on 17 HH patients showed that subjects free of T2D and liver cirrhosis exhibited normal insulin sensitivity and decreased insulin response (25). Additionally, a meta-analysis of 11 prospective studies including patients with HH showed that increased body iron stores were considerably associated with a greater risk of T2DM (26). Consequently, it is thought that iron-related diseases such as HH, β thalassemia and high iron levels in the body are more likely related to the development of insulin deficiency and T2D.

1.3. Iron overload and insulin deficiency

In metabolically highly active cells, such as β cells and neurons, excess iron is believed to be particularly deleterious, probably due to the high production of mitochondrial free radicals. There is substantial evidence illustrating increased iron stores being predictive of insulin deficiency and, consequently, T2D development and iron depletion being protective (11). Although the underlying mechanism of iron-induced insulin deficiency is uncertain, it is likely to be mediated by three fundamental mechanisms: 1) the role of iron in mitochondria, 2) effects of ROS on apoptosis, and insulin gene transcription, 3) insulin resistance.

Iron plays several critical roles in maintaining the physiological function of pancreatic islet β cells (27). First, in mitochondria, iron plays a crucial role in the electron transport chain to generate ATP synthesis, serving as the proximate trigger of glucose-stimulated insulin secretion. Glucose enters β cells and undergoes glycolysis before entering the mitochondria, resulting in increased ATP production (27). Increased ATP: ADP ratio triggers the stimulus-secretion coupling and insulin secretion, while AMPK has been accepted as a negative regulator of insulin secretion. However, excess iron accumulation in β cells leads to reduced ATP production and increased AMPK activity, possibly due to free iron binding to ADP (Figure 1.1) (28). These ultimately cause decreased insulin secretion. Mitochondrial iron overload disease, known as Friedreich's ataxia, an inherited neurodegenerative disorder caused by reduced frataxin production, is associated with the mitochondrial inner membrane and the formation of Fe-S clusters (29). Excessive iron in Friedreich's ataxia patients leads to mitochondrial DNA damage, increased oxidative stress, high AMPK activity, and defective insulin secretion. Friedreich's ataxia is thus associated with a high risk of T2D (30). Another disorder of iron-related mitochondrial DNA, Wolfram syndrome, a mutation in mitochondrial iron import regulator Miner1, is associated with a mitochondrial mutation (tRNA) and, consequently T2D (31).

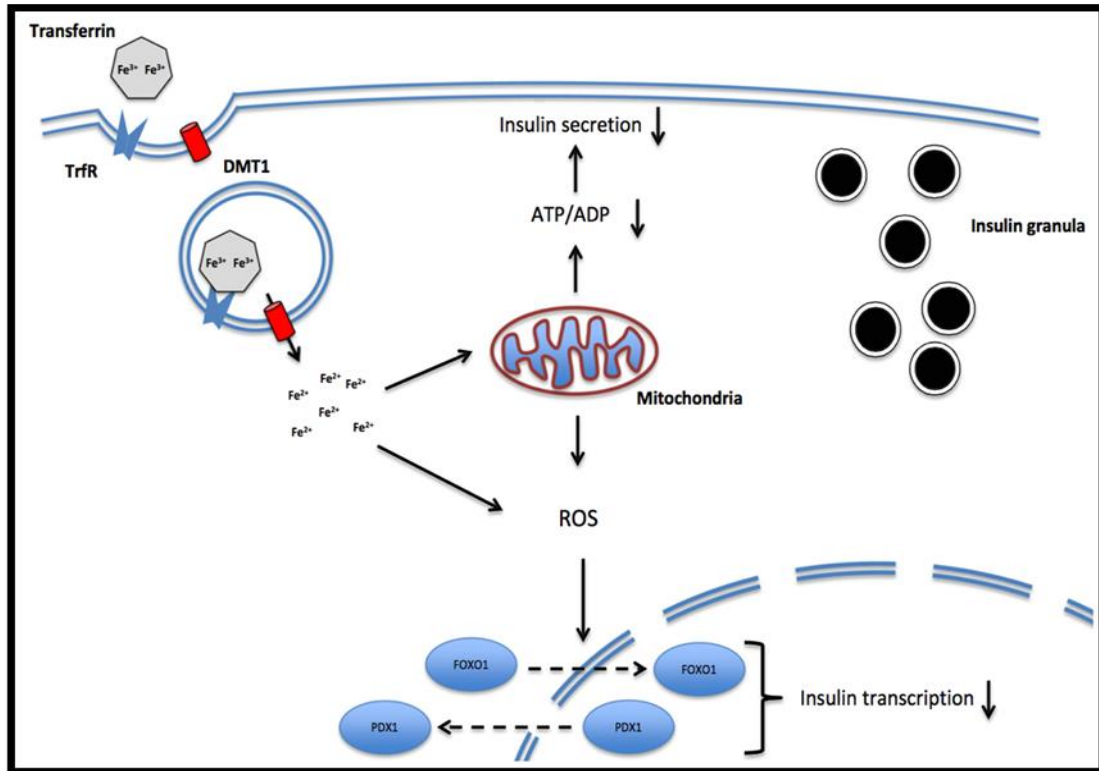


Figure 1.1. Pathological effects of iron on insulin secretion in pancreatic β cells (27). Accumulated iron in β cell mitochondria leads to mitochondrial dysfunction and thus inhibiting the generation of ATP, and ultimately insulin secretion. Besides, excess iron generates ROS through Fenton reactions and functions of mitochondria, causing a halt in insulin transcription.

Pancreatic β cells are extremely vulnerable to reactive oxygen species (ROS) activity resulting from perturbations in iron homeostasis, suggesting that the expression of antioxidant enzymes is relatively low in pancreatic islets. Moreover, compared to the other tissues, pancreatic islets have low ferroportin expression and high DMT1 expression, which might render them more susceptible to elevated levels of free serum iron (32). It is thought that increased ROS formation is more likely to trigger pancreatic β cell apoptosis. As a powerful pro-oxidant and catalyst that promotes the formation of hydroxyl radicals, iron can attack pancreatic β cells through increased oxidative stress, resulting in activation of the apoptotic pathway, and β cell death (33). Oxidative stress is associated with an imbalance between the production of ROS and the antioxidant capacity of the cell. ROS can mediate translocation of the nuclear factor κ B (NF- κ B) from the cytoplasm to the nucleus, thereby inducing the transcription of pro-apoptotic genes (Figure 1.1). NF- κ B belongs to a family of transcription factors that controls the regulation of cell proliferation, differentiation, inflammation,

immune response, apoptosis, and cardiac hypertrophy. ROS thus activate the intrinsic apoptotic pathway leading to permanent β cell loss (27). Another role of iron-accelerated ROS generation is to impair insulin gene transcription, leading to decreased insulin synthesis. Fenton reaction generated-ROS cause cytoplasmic-to-nuclear translocation of Foxo1 and nuclear-to-cytoplasmic translocation of the transcription factor PDX-1, impeding insulin transcription (28). Foxo1, insulin encoding gene, binds to the promoter region of the Ins2 (Insulin II) gene, therefore causing low insulin transcription (Figure 1.2) (27). A decreased level of insulin implicates an increased risk for T2D as glucose uptake is hampered. Besides, ROS can translocate NF- κ B from the cytoplasm into the nucleus to block the transcription of insulin, pancreatic β cells thus cannot properly secrete insulin (Figure 1.2) (34).

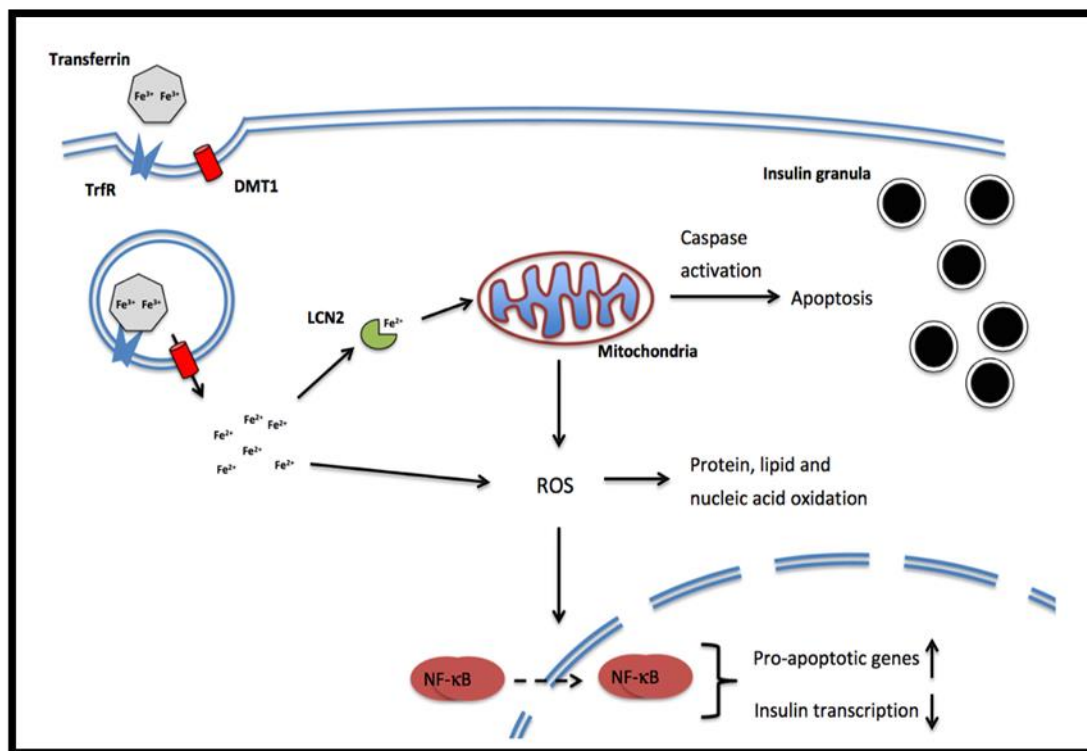


Figure 1.2. Possible mechanism of iron-induced pancreatic β -cell death (27). Excess intracellular β cell iron induces ROS production through Fenton reaction. Increased ROS can mediate translocation of NF- κ B, decreasing insulin transcription.

On the other hand, iron principally generates ROS to control glucose oxidation and insulin secretion. It should be emphasized that low levels of ROS are essential for normal cell signalling molecules that mediate cellular differentiation, survival, and metabolism (35). Iron has the precise ability to cycle reversibly between Fe^{2+} and Fe^{3+} states, which is essential for normal function. Nonetheless, it could be hazardous since catalytic iron converts poorly

reactive free radicals, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{OH}^\bullet$) into highly reactive ones, such as $\text{O}^\bullet$, $\text{O}_2^\bullet$. Moreover, catalytic iron contributes to the amplification of injury caused by free radicals and modulate several steps involved in inflammatory lesions. Previously, Baliga *et al.* (36) have demonstrated that there is a weak correlation between catalytic iron and total body iron stores to show acute renal failure. Thus, it is essential to measure iron pools consisting of chemical forms that can participate in redox cycling (catalytic iron) to point out the association between iron and insulin deficiency, T2D.

Insulin deficiency also appeared to be closely linked to insulin resistance syndrome. Insulin is an endocrine hormone, which promotes glucose disposal in muscle and adipose tissue, and prevents glucose production by the inhibition of glycogenolysis and gluconeogenesis in the liver (37). In insulin resistance states, normal circulating levels of insulin are inadequate to elicit a healthy insulin response in fat, muscle, and liver cells. Therefore, pancreatic β cells begin to secrete more insulin to overcome the hyperglycaemia in tissues. Overtime, pancreatic β cells are not able to produce sufficient insulin to correct worsening tissue insulin requirements; thus, hyperglycaemia occurs, and results in T2D (38). Excess iron that increases the cell oxidative stress leads to inhibition of insulin internalization and actions, resulting in hyperinsulinemia and insulin resistance. Both iron-related oxidative stress and insulin resistance cause endothelial and tissue damage, and finally permanent pancreatic β cell loss (17,38).

Interestingly the relationship between iron and insulin is bi-directional, where excessive iron interferes with insulin inhibition of glucose metabolism, and insulin affects several iron metabolic pathways. Regarding iron metabolism, the hyperinsulinemic situation favors the intrahepatic deposition of iron. Insulin enhances the uptake of extracellular iron in adipose tissue and liver, inducing the redistribution of TfRs to the cell surface while downregulating hepcidin expression. This effect of insulin on the upregulation of iron uptake occurs concurrently with its impact on glucose uptake (17).

1.4. Ferroptosis

A growing body of pathological evidence indicates that iron overload plays an essential role in programmed cell death by activating oxidative injury (39). Ferroptosis is a form of regulated cell death based on the iron-dependent accumulation of lipid hydroperoxides to lethal levels, thereby leading to pancreatic cell death following insulin resistance and T2D (40). Interestingly, ferroptosis is dependent upon intracellular iron accumulation, but not that of other metals, and this form of iron-dependent cell death is distinct from other types of regulated cell deaths at the morphological, biochemical, and genetic levels (41).

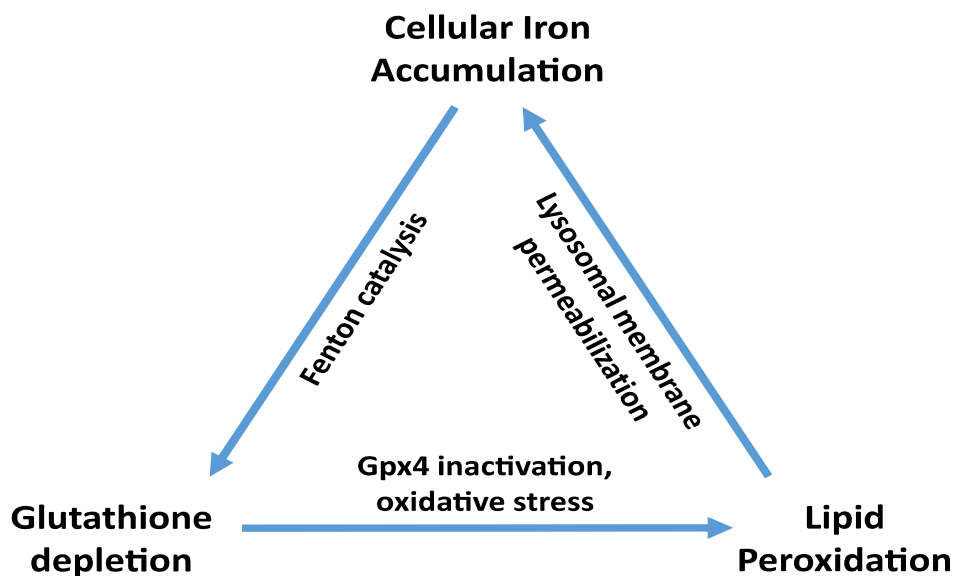


Figure 1.3. Summary of hypotheses of ferroptosis (42). Iron accumulation, glutathione depletion and membrane lipid peroxidation are conditions to cause ferroptosis.

Ferroptotic cells exhibit altered mitochondrial morphology and cristae structure. It is characterised morphologically by the presence of smaller than usual mitochondria with condensed mitochondrial membrane density and reduction of the mitochondria crista (43). Additionally, induction of ferroptosis by inactivation of glutathione peroxidase 4 (GPX4), an antioxidant enzyme that maintains redox status and oxidative stress, has genetically and biochemically been associated with outer mitochondrial membrane rupture (44,45). Ferroptosis can be triggered by some molecules such as erastin, RSL3, and ML-210, which can inhibit cell growth and alter the mitochondrial phenotype in the presence of iron, and it is also significantly inhibited by both antioxidants and iron chelators (46). In in vitro studies, erastin is a significant chemical to initiate and mediate conditions associated with ferroptosis

(46,47). These conditions are more likely to be related to: (i) accumulation of free iron, (ii) depletion of the antioxidant GSH, and (iii) lipid peroxidation (42). Erastin contributes to iron accumulation within cells, even though the role of iron in ferroptosis is unclear (42,47). It was hitherto hypothesised that the principal role of iron in ferroptosis was free radical production. Iron is a pro-oxidant because it converts hydrogen peroxide to hydroxyl radicals via the Fenton reaction, thus inducing cell damage (48). Secondly, erastin can act as a system X_c^- inhibitor to initiate ferroptosis. System X_c^- is an aminoacid antiporter that typically mediates the exchange of extracellular cysteine and intracellular glutamate across the cellular plasma membrane, thus ensuring maintenance of intracellular GSH levels (Figure 1.4) (42). One metabolic consequence of system X_c^- inhibition resulting from erastin is depletion of the intracellular cysteine amounts, which is a precursor for GSH synthesis, thus contributing to ferroptosis as shown in Figure 1.4 (40). In addition, fatty acids are vulnerable to oxidation from Fenton radicals, involving enzymes such as lipoxygenases and cyclooxygenases, and the lipid peroxidation chain reactions. Endogenous free radicals that damage lipid membranes lead to lipid peroxidation, suggesting that lipid peroxidation products such as malondialdehyde (MDA) and 4-hydro-2-nonenal are increased during the ferroptotic process (48,49).

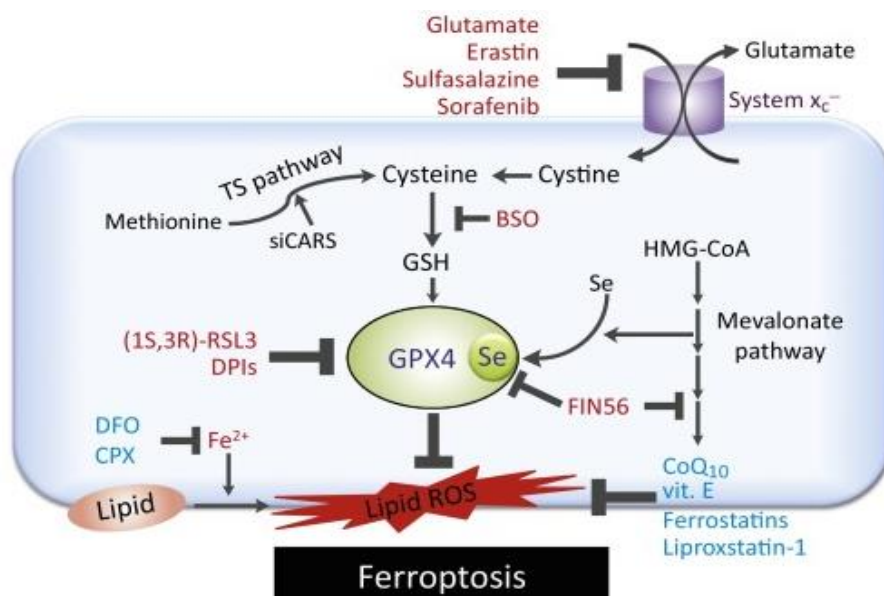


Figure 1.4. Molecular pathways of ferroptosis regulation (48). Ferroptosis inducers block system X_c^- , thereby reducing GSH production and lack of GPX4 activity. Additionally, excess intracellular iron produces ROS through Fenton reaction. Eventually, membrane lipid peroxidation occurs, thus leading to ferroptotic cell death.

1.5. Treatments of iron overload

1.5.1. Chelators

Iron overload is a severe clinical condition that can be largely prevented using iron-specific chelating agents, such as deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX) (50). In order to avoid deleterious complications of iron overload, it is necessary to suppress plasma iron and remove excess iron. In major β thalassemia and HH, phlebotomy is impossible because patients are anaemic. Thus, the best selection for the treatment of iron overload is iron chelation therapy. The successful design of a non-toxic, orally active, selective iron chelator has become a critical sought-after goal. In order to determine an ideal iron chelator for clinical use, several specifications must be considered, such as metal affinity and selectivity, kinetic stability of the complex, toxicity, and bioavailability (51).

The aim of chelation therapy prevents the accumulation of excess iron and its complication, such as pancreatic dysfunction, which results in impaired insulin secretion. Studies in HH patients whose body iron stores were reduced with iron chelation therapy resulted in improved glycemic control, and 30-40% of patients managing to reduce dependency on oral hypoglycemic therapy (51). In 1989, Cutler administered deferoxamine to nine T2D patients with hyperferritinemia who were negative for the most common hemochromatosis haplotypes. Major improvement in the metabolic control was observed with seven patients, and parallel reductions in baseline concentrations of glucose, triglycerides and glycated hemoglobin and treatment with either insulin or oral hypoglycemic agents could be discontinued in some patients (52). Additionally, the use of a DFO/DFP combination for 24–36 months resulted in an improvement of insulin resistance and impaired glucose tolerance in patients with diabetes in Fe-loaded thalassemic patients (53) .

However, the side effects of long-term iron chelation therapy should be taken into consideration in iron overloaded patients. The toxicity associated with iron chelators originates from several factors; including inhibition of iron-containing metalloenzymes; lack of metal selectivity, which may lead to the deficiency of other physiologically important metals such as zinc (II); redox cycling of iron-complexes between Fe^{+2} and Fe^{+3} , thereby generating free radicals and the kinetic lability of the iron-complex leading to iron

redistribution (54). Thus, alternative iron chelation treatments should be investigated for iron overload disease.

1.5.2. Phenolic Compounds

Polyphenols are known as abundant micronutrients in the human diet, and evidence for their role in preventing degenerative disorders such as cardiovascular diseases and cancer is emerging. The health effects of polyphenols depend on the amount consumed and, on their bioavailability (55). Phenolic compounds are secondary metabolites of plant origin foods that include a significant number of structurally diverse compounds that have a polyphenol structure, comprising several hydroxyl groups on aromatic rings, but also molecules with one phenol ring such as phenolic acids and phenolic alcohols. The term of phenol can be construed as compounds that possess an aromatic ring bearing one hydroxyl group. In contrast, polyphenols can have one or more aromatic rings bearing more than one hydroxyl group (55,56).

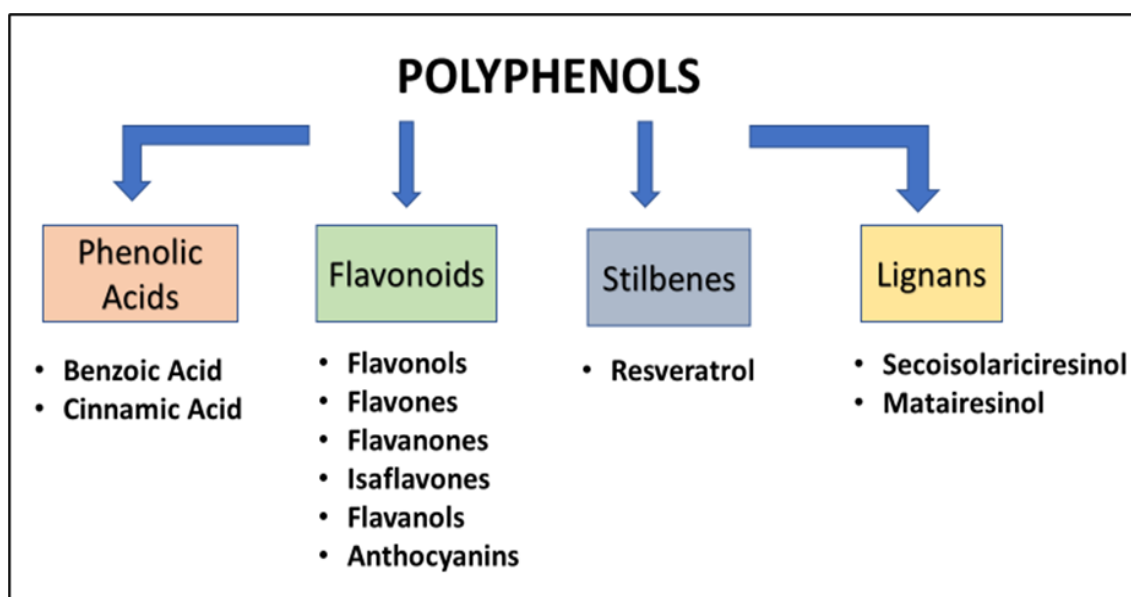


Figure 1.5. Classification and chemical structure of polyphenols (56). Polyphenols comprise; phenolic acids, flavonoids, stilbenes and lignans.

In the past decades, plant-origin polyphenols have drawn increasing scientific attention due to their significant antioxidant and other properties and their vital effects in preventing various oxidative stress-associated diseases (57). Two mechanisms are commonly proposed to explain the multifunctional antioxidant role of phenolic compounds: metal chelation and free radical scavenging. Therefore, plant polyphenols can interfere not only with the

propagation reactions of the free radicals by means of scavenging them but also with the formation of radicals by chelating the transition metal ions (58). Numerous authors have investigated the free radical scavenging activity of plant polyphenols. Polyphenols react with ROS to form relatively stable phenolic oxygen radicals, thus reducing free radicals. The electrons on the benzene ring in polyphenol structure have a conjugation effect on the single electron on the oxygen atom of the phenolic hydroxyl group. The single electron tends to attach the benzene ring, thus limiting the activity of the hydrogen-oxygen bond in the phenolic hydroxyl group. The hydrogen activity on the phenolic hydroxyl group raises, and the free radicals compete for active oxygen, limiting the auto-oxidation reaction of the free radicals. Polyphenols also have a certain different degree of antioxidant capacity (59); however, there is a difference in eliminating free radicals due to the structural differences. In general, the ability of polyphenols for scavenging ROS is associated with the number of hydroxyl groups in the environment, the structure, and the stability of the phenolic oxygen radicals (60).

Several reports have elucidated the iron or copper metals chelating ability of polyphenols, kinetics, and mechanisms of reactions of polyphenols with ferric iron, superoxide radical scavenging by polyphenol iron complexes, chemoprotective effect, and the binding constants of complexes. However, the interaction of plant polyphenols with transition metal ions has not been fully resolved (61). Polyphenolic compounds have several chemical moieties, and it has been suggested that iron chelation can take place when (i) ortho-dihydroxyl groups; (ii) C5-OH and/or C3-OH moieties in conjunction with a C4 keto-group; and (iii) a large number of OH groups are present (62). A study by Messner *et al.* (63) showed that curcumin treatment reduced iron-dependent oxidative stress and iron toxicity in T51B rat liver epithelial cells exposed to 500 mM FAC. Also, in mice fed high-iron diets, curcumin treatment decreased the H and L subunits of the iron storage protein ferritin, increased TfR1, and the activation of iron-regulatory protein (IRP) in the liver (64). Caffeic acid, a primary phenolic compound of the diet, inhibited liver oxidative stress induced by iron overload in male Wistar rats (65), suggesting that diet rich in phenolic compounds might prevent iron-related hepatotoxicity.

Therefore, polyphenols are thought to play a naturally protective role in excess iron-related diseases and iron-induced ROS generation. Treatment of iron overload is associated with iron chelators, which are hampered by their off-target and side effects. For example, DFP and DFX

are chemical chelators; however, the former increases the risk of agranulocytosis, whereas the latter increases the risks of gastrointestinal disturbances and hepatic failure. These therapeutic limitations of currently available iron chelators highlight the need for natural interventions such as polyphenols (66). In this study, curcumin and EGCG were used to analyse their antioxidant and chelating features in MIN6 cells based on the several in vitro and in vivo studies on them.

The most abundant curcuminoid compound in turmeric, curcumin is the most prevalent polyphenol in the Western Diet. Consumption of curcumin has been estimated at 80-180 mg/day, which is not associated with adverse effects in humans (67). Curcumin has been safely administered at high doses in many clinical trials, has achieved the status of “Generally Recognized as Safe” by the US Food and Drug Administration (FDA)(68). Bioavailability of curcumin is low, which could limit its health benefits despite of wide range of biological effects. The main reasons for its low plasma and tissue concentration are its poor absorption, rapid metabolism, and rapid systemic elimination (69).

When curcumin is consumed in the diet, a significant proportion is excreted in the faeces and only a small portion is absorbed in the intestinal system. The absorbed curcumin is rapidly metabolised in the livers and plasma (70). Curcumin is converted to its water-soluble metabolites (glucuronides and sulphates) and excreted through urine. The metabolism of curcumin is composed of two phases. Phase I metabolism is a NADPH-dependent reduction. It comprises the reduction of the double bonds of the heptadiene-3, 5-dione structure catalysed by NADPH-dependent curcumin or dihydrocurcumin reductase. In phase II metabolism, curcumin is conjugated to sulphates and glucuronides in the liver (71). Liver can be indicated as the primary organ responsible for the metabolism of curcumin. Holder *et al.* (72) reported that the primary metabolites of curcumin are glucuronides of tetrahydrocurcumin in rats. In addition to glucuronides, sulphate conjugates were also found in the urine of curcumin-treated rats. The concentration of curcumin conjugates in human plasma under normal physiological conditions is in the range 0.5-2 μ M (69,71).

Curcumin alleviates oxidative stress via several mechanisms. Three reactive functional groups of curcumin are responsible for its direct radical scavenging property: two phenolic groups and one diketone moiety which are hydroxyl, methoxy and 1,3-diketone conjugated diene system in curcumin structure (Figure 1.6). The specific metal chelator effect of curcumin was

established previously in cell free experiments and was attributed to the presence of chemical groups such as β -diketonate group, suggesting that curcumin can make an iron complex in the ratio of 1:1 (72). Also, curcumin upregulates antioxidant enzymes and iron-related proteins by triggering nuclear factor erythroid 2-related factor 2/antioxidant response element signalling pathway suggesting that curcumin may act as an antioxidant compound by its iron-binding activity, inducing IRP proteins (73). Some animal and in vitro studies have demonstrated that curcumin has iron-chelating activity and leads to iron depletion (74,75). It has been indicated that excess iron is responsible for functional abnormalities during iron overload syndrome; thus, curcumin supplementation can provide beneficial effects during iron overloaded issues by chelating with free iron (66).

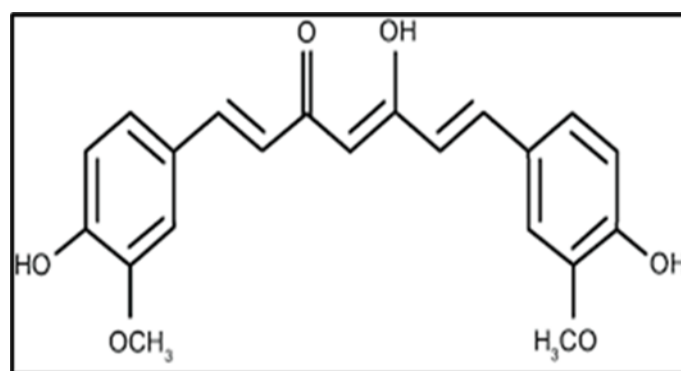


Figure 1.6. Chemical structure of curcumin (70)

(-)-Epigallocatechin gallate (EGCG) is the most abundant and biologically active catechin. It accounts for almost 50–75% of the total amount of catechins. Furthermore, EGCG constitutes approximately 40% in the polyphenols group (76). EGCG appears to be the most effective constituent of green tea, a widely used traditional health drink. Figure 1.7 shows the chemical structure of EGCG. The bioavailability of ingested EGCG is relatively low micromolar range in human subjects (77). It has been shown that the maximum plasma concentration of EGCG was only 0.15 μ M in human subjects administrated two cups of green tea (78). The major factors decreasing the intestinal absorption of oral EGCG are as follows: the low stability of EGCG in the intestinal system (79), and the poor intestinal absorption (80). The approximate daily intake of EGCG through green tea consumption from the diet reach approximately 560 mg/day. The European Food Safety Agency (EFSA) reviewed that daily intake of EGCG should not be above 800 mg. Nakagawa and Miyazawa (81) reported that the highest concentration of EGCG in human plasma and fasting rats was 1047 mg/L (equivalent to

0.012% of EGCG intake) and 156 mg/L (equivalent to 0.32% of EGCG intake), respectively. Additionally, EGCG is detected mainly in the form of methylation or sulfation conjugates in the plasma and urine, while in the liver, it shows EGCG glucuronidation complexes. The effective concentrations of EGCG ranged from 1 to 100 $\mu\text{mol/L}$ in vitro studies (82).

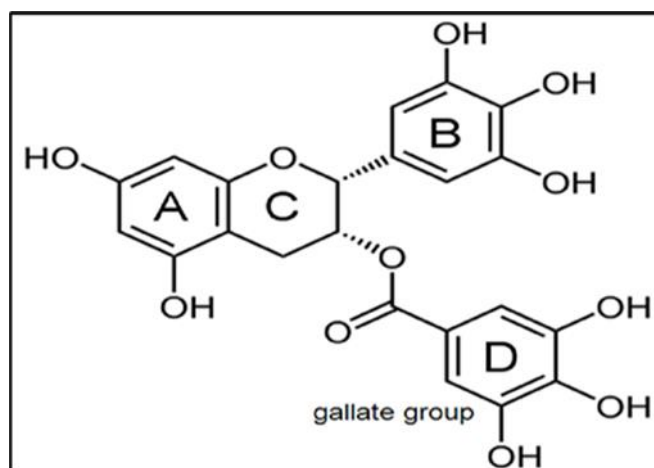


Figure 1.7. Chemical structure of (-)-epigallocatechin gallate (EGCG) (82)

A great deal of attention on EGCG is received as a therapeutic alternative for treating some diseases, which might restore the balance between the natural antioxidants and free radicals by direct eliminating of ROS and by enhancing the activity of natural antioxidant enzymes (83) and metal chelation activity (84). This ability is primarily attributed to the 3 phenolic rings in structure of EGCG and their multiple hydroxyl groups (85). In vitro studies showed that EGCG possesses antioxidant properties and favourable effects on gene expression, signal transduction, and other cell functions (86). Molecular studies demonstrated that EGCG might interact strongly with ferritin with a high binding affinity (87).

Phenolic Acids

Phenolic acids commonly occur in foods and form approximately one-third of the total intake of plant polyphenols, the most abundant antioxidants in the human diet. Phenolic acids are hydroxyl derivatives of aromatic carboxylic acids, which have a single phenolic ring and can be divided into two main types, the benzoic acids and cinnamic acids (88). Ferulic acid (FA) is the most abundant phenolic acid found in cereal grains, which constitutes its primary dietary source. FA is more easily absorbed into the body and stays in the blood longer than any other phenolic acids; thus, it is considered to be a superior antioxidant (89). Ferulic acid has low toxicity and possesses several physiological functions, including anti-inflammatory, antimicrobial, anticancer anti-arrhythmic, and antithrombotic activity. It also demonstrated

antidiabetic effects and immunostimulant properties, and it reduces nerve cell damage and may help to repair damaged cells (89). Then, this study tested ferulic acid and its metabolite FAS to show their antioxidant and metal chelation features in vitro (Chapter 4) and in vivo (Chapter 5) because curcumin and EGCG could not show expected antioxidant activity on Nrf2 pathway.

FA is abundantly distributed in plants, seeds, grains, fruits, and wheat, such as coffee 9.1–14.3 mg/100 g, whole brown rice 42 mg/100 g, corn flour 38 mg/100 g, wheat 74 mg of FA/100 g, popcorn 313 mg/100 g, sugar beet pulp 800 mg/100 g and barley extract 1358–2293 mg/100 g. Dietary consumption of FA has been estimated at 150-250 mg/day (90).

Compared with other phenolic acids, FA is freely cell-permeable and possesses high bioavailability in rats. More than 70% of ingested FA is quickly absorbed in free form through the gastrointestinal tract and is transported into liver where approximately 6% of FA may be conjugated with glucuronide or sulphate. Subsequently, remaining free and conjugated FA complexes enter the systemic circulation, and approximately 50% of FA is distributed and eliminated via the kidney (91). This phenolic compound remains in the circulation longer than other naturally occurring compounds like vitamin C; however, FA is expected to stay in the circulation long enough to elicit its effects. The concentration of FA in plasma under normal physiological conditions is in the range 0.2-2 μ M FA (92).

After oral administration of FA in human, its six sulphate metabolites were detected in the plasma, including: ferulic acid-4'-O-sulfate, dihydroferulic acid-4'-O-sulfate, vanillic acid-4-O-sulfate, 3-phenylpropionic acid-O-sulphate, 3',4'-dihydroxyphenylacetic acid and hippuric acid (93). Metabolites of other phenolic acids are shown to exert significant biological activities comparable to or higher than their parent compounds (94). In urine samples of human subjects, sulphated form of FA is the predominant form of FA. It is suggested that the sulphated metabolites of FA compared to the free form of FA can be related to different effectiveness in cellular uptake and cell internalization (95). It was observed that FA-4-O-sulfate is more bioactive than FA in lowering blood pressure in mice. However, there are no data supporting that sulphated metabolites of FA have more potent bioactivity than its native compound.

In vivo FA administrations ranged from 10 to 300 mg/kg to show antioxidant effects in tissues. In vitro, concentration of 10–150 μM of FA counteracted nicotine-induced lipid peroxidation and glutathione depletion in lymphocytes (96). Moreover, at concentrations of 250–500 μM FA reduced protein and lipid peroxidation induced by reactive oxygen species in neuronal cells and peripheral blood cells (97). This evidence of beneficial effects has only however been investigated in vitro and in rodents. The dose used in several the studies is over the estimated human intake and consists of 100% free FA (98).

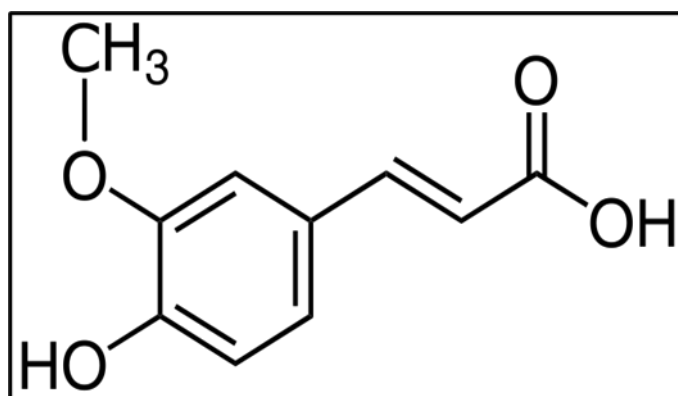


Figure 1.8. Chemical structure of ferulic acid (98)

FA possesses three distinct structural motifs that are more likely to contribute to the free radical scavenging capability. The presence of electron-donating groups, which are 3-methoxy and 4-hydroxyl in FA, provides a stabilised radical that scavenges ROS and chelate metal ions. Ramar *et al.* (98) investigated the protective effects of FA in alloxan-induced diabetic mice, and oral administration of FA significantly decreased glucose concentrations, lipid peroxidation markers, and increased levels of antioxidants, SOD, and CAT. In addition, FA administration to Wistar albino rats treated with formaldehyde, a very reactive toxic chemical, markedly increased CAT, SOD, and GSH activities and caused a remarkable decrease in MDA concentrations in the liver. Thus, suggesting that FA can be used as a promising hepatoprotective agent against oxidative stress-related diseases because of the apparent beneficial effects on oxidative stress parameters (99). Another functionality of FA is the carboxylic acid group with the unsaturated carbon-carbon double bond that can contribute to the stability of the free radicals by providing additional reactive sites for these free radicals. In addition, this functionality will likely facilitate the anchoring of FA into lipid bilayers by protecting against lipid oxidation. Therefore, it possesses an antioxidant property by its phenolic hydroxyl group and carboxylic acid group (100). A study by Sanjeev showed that FA

treatment consumption ameliorated the cadmium-induced liver and renal lipid peroxidation levels in Wistar albino rats due to a decrease in the amount of cadmium in these tissues (101). Similar results were obtained by Zhang *et al.* (102) that FA treatment in cerebellar granule cells exposed to 200 μM of FeSO_4 decreased a level of the oxidative marker, lipid peroxidation level, binding free iron to prevent them from oxidative damage.

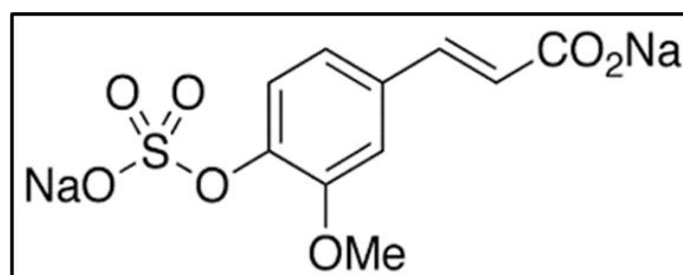


Figure 1.9. Chemical structure of Ferulic Acid 4-O-Sulfate Disodium Salt (FAS)(95)

Therefore, it is thought that FA can improve oxidant-antioxidant balance, enhance the endogenous antioxidant system, and effectively prevent oxidative damage; FA is the most abundant phenolic acid, and FA was studied in this PhD project to elucidate its antioxidant and chelation properties against iron-related damage by activating antioxidant pathway.

1.6. Nrf2 Antioxidant mechanism, activation, and diseases

1.6.1. Nrf2 Pathway

Cells must cope with several oxidative xenobiotic and electrophilic stressors apart from iron-mediated oxidative stress (103). Therefore, there are a variety of cellular anti-stress mechanisms to ameliorate these toxic exposures. One vital mechanism is the Kelch-like ECH-associated protein 1–nuclear factor (erythroid-derived 2)-like 2/Antioxidant response element (KEAP1-Nrf2/ARE) (104).

This mechanism works on the basis that under basal or unstressed conditions, homodimers of KEAP1 negatively regulate the Nrf2 transcription factor by targeting it for ubiquitination and subsequent proteasomal degradation. However, following electrophilic and oxidative stress, cysteine residues on KEAP1 are modified; thereby inducing a conformational change in KEAP1, disrupting its interaction with Nrf2 and thus preventing Nrf2 degradation (104). Newly synthesized Nrf2 accumulates, translocates to the nucleus, dimerizes with small musculoaponeurotic fibrosarcoma (MAF) proteins, and facilitates transcription of Phase II metabolism and cytoprotective genes. For example, Nrf2 target genes include NADPH

quinone dehydrogenase 1 (NQO1), glutamate–cysteine ligase modifier subunit (GCLM), and the peroxide-scavenging selenoproteins of the glutathione peroxidase (GPX) family (Figure 1.10) (105).

Genetic deletion of transcription factor Nrf2 renders cells and animals much more sensitive to the damaging effects of oxidants, electrophiles, and inflammatory agents in comparison with their wild-type counterparts (105,106). On the contrary, activation of the Nrf2 pathway allows survival and adaptation under various conditions of stress and has protective effects in in-vivo models. Cross-talks with other signalling pathways have elucidated the role of the Nrf2 pathway in determining the fate of the cell, impacting fundamental biological processes such as proliferation, apoptosis, angiogenesis, and metastasis (105,106).

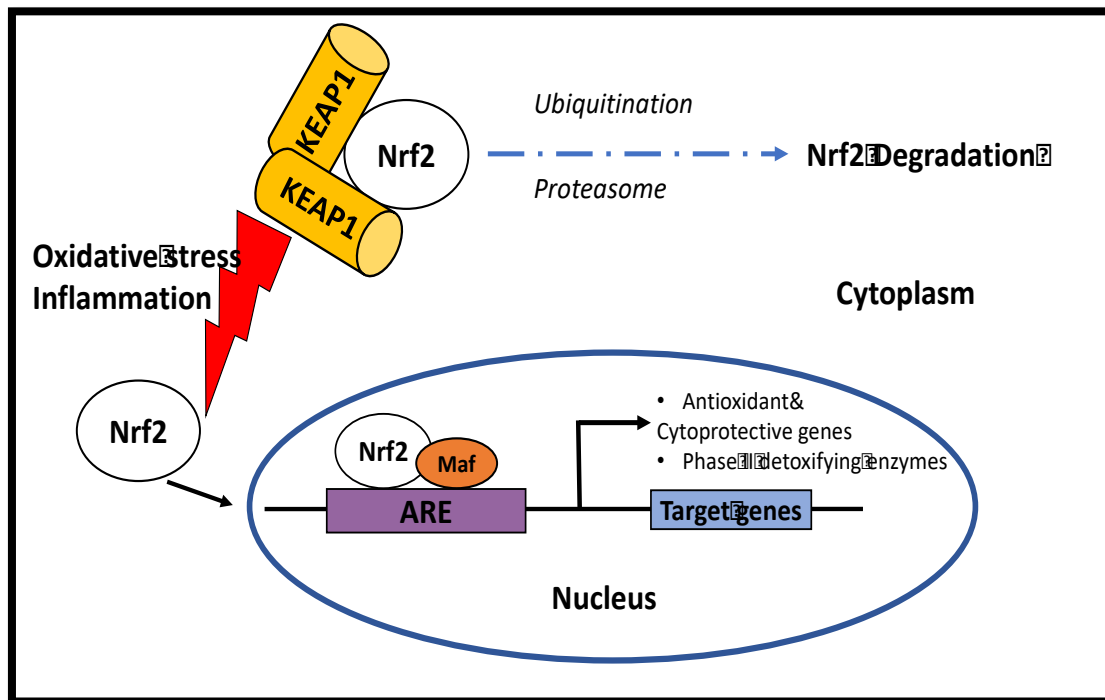


Figure 1.10. Schematic diagram of the Nrf2-Keap1/ARE signalling pathway (Adapted from (107)). Under normal conditions, Nrf2 is constantly ubiquitinated through Keap1 and degraded in the proteasome. After exposure to oxidative stress or inflammation, Keap1 is inactivated and Nrf2 becomes phosphorylated. Phosphorylated Nrf2 accumulates in the nucleus and binds to ARE sites, subsequently activating many genes including antioxidants, detoxifying enzymes.

1.6.2. Nrf2 Activation by Natural Products

Natural products have been accepted as an efficient resource for the discovery of potential therapeutic agents. Liao *et al.* (107) reviewed the main role of various natural polyphenols as medicine and food in disease prevention. At present, the exploration for leading compounds in natural products with therapeutic function and the further optimization for their structures has become an essential approach in modern medicinal chemistry research. With the steady improvement in the technologies used in the isolation and purification of monomer components of natural products, specific individual ingredients of natural products have been drawing attention as Nrf2 activators due to their excellent efficacy (107).

Several natural polyphenols, inhibiting the production of ROS, increasing Nrf2 nuclear translocation and DNA binding ability, interfering with Keap1-Nrf2 interaction, and promoting Keap1 ubiquitination, ultimately maintain the physiological balance of the body (107). Although many polyphenolic compounds could induce ARE activation, and a few enhance Nrf2 expression or nuclear translocation, the precise mechanism by which these polyphenols influence the Nrf2-Keap1 complex to activate ARE is poorly understood (107). A study revealed that epigallocatechin gallate (EGCG) is a Nrf2 potent activator polyphenol with multiple hydroxy groups. Studies on EGCG-treated fluoride-poisoning rats showed significantly enhanced activation of Nrf2 and its target genes HO-1, GCLC, and NQO1, and highly inhibited Keap1 in the testis and thus leading to significant normalization of oxidative stress markers, sperm function, testosterone, and antioxidant status (108).

Caffeic acid, a phenolic compound, can be used as an electrophile in addition to its nucleophilic nature, which can provide Nrf2 translocation and transcription (109). In the study by Pang *et al.* (110), it was demonstrated that caffeic acid inhibited the binding of Keap1 to Nrf2 by decreasing Keap1 expression, thereby activating Nrf2 and leading to increased expression of antioxidant signals and reduced cellular ROS levels including HO-1 and NQO1 to prevent acetaminophen-induced acute liver failure in human healthy liver L-02 cells and HepG2 cells. FA is a rich monomer ingredient that is an abundant phenolic acid in vegetables. There is a methoxy group on the benzene ring of FA, which makes it a strong ROS scavenger (89). In the study by Catino *et al.* (111), FA regulated the Nrf2/HO-1 system and counteracted trimethyltin-induced neuronal disorder in the human neuroblastoma cell lines SH-SY5Y. In

addition, FA abrogated oxidative stress and DNA damage by up-regulating nuclear translocation of Nrf2 in Swiss albino mice exposed to γ -radiation (112).

Clinical investigations of Nrf2 activation through natural compounds indicated their benefits in the prevention of oxidative stress. Further research needs to include the potential confounders such as ethnicity, dosage, subject age, and treatment length to firmly establish the role of Nrf2 activation.

1.6.3. Modulation of Ferroptosis by Nrf2 mechanism

Nrf2 activation has been linked to protection against ROS related lipid peroxidation (113). Namely, Nrf2 regulates ferroptosis in many ways, and the relationship between the key pathways for ferroptosis regulation and Nrf2 has already attracted attention. There are two main approaches to dissect Nrf2 antioxidant pathway against ferroptosis: iron metabolism and GSH metabolism (114).

With regards to GSH homeostasis, system Xc⁻ or SLC7A11 antiporter is directly related with GSH synthesis, which exports glutamine and imports cystine into the cell to produce GSH. Lin *et al.* (115) found that down-regulating SLC7A11 expression can result in decreased cystine-dependent glutathione peroxidase activity, cellular antioxidant capacity, lipid activity and oxygen elevation, which eventually causes cellular ferroptosis (115). Thus, GSH is used by GPX4 to reduce lipid peroxides to their alcohol form, an important step in preventing ferroptosis. Aberrant accumulation of lipid peroxides through inhibition of GPX4 or depletion of GSH has been shown to induce ferroptosis, an iron-dependent and nonapoptotic form of cell death after deletion of SLC7A11 (116). Also, blocking SLC7A11 in HT-1080 cells treated with erastin leads to depletion of GSH and thus cell death. The activation of Nrf2-Keap1 complex increases system Xc⁻ (SLC7A11) and promotes glutamate secretion (117). Yoo *et al.* (118) showed that GPX4 deletion in adult mice alleviated the activity of hippocampal neurons and astrocyte proliferation, thus this change is more likely to link Alzheimer's Disease to ferroptosis (118).

In addition to the regulation of system Xc⁻, Nrf2 pathway related genes are involved in the maintenance of redox homeostasis, operating iron, and haem metabolism. They regulate haem-oxygenase 1 (HO-1), ferroportin (FPN), and light chain and heavy chain of ferritin (FTL/FTTH1) (119). In other words, activation of Nrf2 could be considered a negative ferroptosis

regulator since it is more likely to activate antioxidant elements and iron storage and suppress cellular ROS production. Cui *et al.* (120) indicated that knockdown of Nrf2 levels by siRNA resulted in greater toxicity of Fe⁺²-induced astrocytes. Targeting the HO-1 regulated by Nrf2 is a cytoprotective enzyme that operates as a key rate-limiting enzyme in the process of degradation of haem to generate ferrous iron, carbon monoxide (CO) and biliverdin (121). The antioxidant effects of HO-1 derive from the degradation of haem, stimulator of ROS production, as well as the ROS scavenging properties of the breakdown products, biliverdin, and bilirubin (121). Dong *et al.* (122) indicated that the transcriptional inhibition of SCL7A11 leads to reduced antioxidant capacity, the accumulation of ROS, the lack of GPX4, and ferroptosis activation. Nrf2 prevents acute lung injury by regulating SLC7A11 and HO-1 that inhibits the effect of pulmonary ferritin (122). In contrast, in Panc-1 pancreatic cancer cells, induction of ferroptosis by artesunate was accompanied by an increase in HO-1 protein accumulation (123), which is associated with the ability of artesunate to increase ROS levels, which in turn activates Nrf2-mediated antioxidant response. Thus, it could be postulated that artesunate induces ferroptosis in pancreatic cancer cells via the HO-1-mediated enhancement of intracellular labile iron which is highly redox active. Also, in murine models, administration of doxorubicin to mice induced cardiomyopathy with a rapid, systemic accumulation of non-haem iron via haem degradation by Nrf2-mediated upregulation of HO-1, which effect was abolished in Nrf2-deficient mice (124). In acute iron exposure, Nrf2 may protect from iron-induced injury, whereas long-term iron exposure resulted in iron accumulation, cytosolic ROS formation, and increased HO-1 mRNA expression, and this was accompanied by nuclear translocation of Nrf2 and induction of its target protein NQO1 (125). Accordingly, this evidence proved the hypothesis that Nrf2 can regulate intracellular iron metabolism through the Nrf2–HO-1 axis pathway to regulate ferroptosis, however, it is important to note that HO-1 has a dual role in ferroptosis.

The transcription factor Nrf2 plays a critical role in the regulation of iron storage and export by activating FTH, FTL and FPN. Excessive iron is stored in ferritin, which is an iron storage protein complex that includes ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1) (119). FTH has iron oxidase activity, which catalyses the conversion of the ferrous form (Fe⁺²) to the ferric form (Fe⁺³) and promotes iron to be safely incorporated into the ferritin shell, thereby reducing free iron levels (119). Liu *et al.* (126) showed that Nrf2 deficiency enhances the

susceptibility of healthy neuronal PC12 cells to ferroptosis. However, the activation of Nrf2 promotes GPX4 activity and iron storage capacity by increasing the level of GSH and the expression of FTH1. Specifically, Nrf2-mediated induction of the iron related target genes HO-1 and FTH1 protected against ferroptosis; consistently, knockdown of either HO-1 or FTH1 by RNA interference enhanced ferroptotic cell death in hepatocellular carcinoma cells (127). Further studies showed that various Nrf2 activators, including 100 μ M diethyl malate and 5 μ M sulforaphane, upregulated FPN mRNA in murine macrophages in an Nrf2-dependent but iron-independent manner, thus releasing intracellular iron (5% extra) to the extracellular milieu by FPN. Nrf2-mediated increases in iron export from macrophages were efficient enough to counteract lipopolysaccharide-induced suppression of FPN mRNA expression. This points out that Nrf2 can regulate iron trafficking during inflammation via FPN expression. Thus, Nrf2 activation might reduce intracellular labile iron amounts by inducing the expression of the iron exporter FPN and iron storage ferritin proteins, FTH1 and FTL (128). Further studies using an in vivo model are vital to show whether alterations to Nrf2-mediated FPN induction might alter infection or inflammation outcomes.

In summary, the results of these studies show that Nrf2 activation modulates lipid metabolism and decrease oxidative damage by regulating iron and GSH metabolisms. Further investigations including human clinical studies are needed to confirm the beneficial effects of Nrf2 activation as supplementary treatments for iron related diseases.

1.7. Aims and Objectives

The overall aim of this project is to investigate the protective function of a phenolic acid, ferulic acid (FA) against iron loading-induced stress in MIN6 pancreatic β cells. As discussed in the introduction, excess iron has been shown to induce oxidative stress due to increased production of ROS. This causes the progressive impairment of β cell function in the pathogenesis of T2D. Consequently, it is vital to study the potentials of natural compounds to alleviate and prevent cell death caused by ROS in a pancreatic cell culture model. Studies were therefore conducted to investigate the potentials of flavonoids, as free radical scavengers, and potent metal chelators to protect pancreatic cells. The mechanism via the Nrf2 activation, by which FA attenuate or reverse the damage caused by iron overload or ferroptosis was also investigated.

The first experimental chapter aims to show if curcumin and (–)- Epigallocatechin-3-Gallate (EGCG) protect murine MIN6 pancreatic β cells against iron toxicity and erastin-induced ferroptosis. This was investigated by:

- demonstrating the inhibition of iron accumulation, and lipid peroxidation by curcumin and EGCG in MIN6 cells.
- determining the antioxidant protective effect of curcumin and EGCG against ferroptosis-induced damage in MIN6 pancreatic cells.

The second experimental chapter aims to determine if FA plays a protective role against ferroptosis in pancreatic MIN6 cells. These aims will be met by:

- determining the protective effects of FA against erastin-induced ferroptosis in terms of the antioxidant activities, such as glutathione levels and the prevention of lipid peroxidation.
- determining if FA can protect pancreatic MIN6 cells against ferroptotic cell death via the activation of the Nrf2 pathway to induce antioxidant proteins, such as the glutamate-cysteine ligase catalytic subunit (GCLC), glutathione peroxidase 4 (GPX4), NAD(P)H quinone oxidoreductase-1 (NQO1) and haem oxygenase-1 (HO-1).

The last experimental chapter aims to investigate how FA prevents tissue and organ damage in a mouse model of iron overload by:

- determining the protective effects of FA and its metabolite, ferulic acid sulphate (FAS) on tissues of iron-loaded BALB/c mouse model.
- investigating the protective effect of FA and its metabolite FAS against iron-induced damage in HepG2 cells to corroborate the in vivo research outcome.

1.8. References

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Chapter 2. General Materials and Methods

2.1. Materials

Polyphenols including curcumin, quercetin, rutin, EGCG, tannic acid and phytic acid were obtained from Sigma-Aldrich Company Ltd (Dorset, UK). Phenolic acids, ferulic acid (FA) and ferulic acid 4-O-sulfate disodium salt (FAS) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). The antibody to NRF2 was obtained from Santa Cruz Biotechnology (Wembley, UK) and GPX4 was purchased from R&D systems (Abingdon, UK). The antibodies to DMT1, FPN, TfR1, NQO1, GCLC, HO-1 and β -Actin were obtained from Abcam (Cambridge, UK). Erastin and ferrostatin-1 were purchased from Bertin Bioreagent (Montigny-le-Bretonneux, France). Deferoxamine, ferrozine and β -mercaptoethanol were purchased from Sigma Aldrich (Dorset, UK). All other reagents were procured from Sigma Aldrich (Dorset, UK) unless specified.

2.2. Methods

2.2.1. Cell culture

The mouse MIN6 pancreatic β -cell line was used in this study (1). The cells (< 30 passages) were routinely cultured in T25-cm² plastic flasks. MIN6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 15% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were kept at 37 °C under a humidified atmosphere containing 5% CO₂. The medium was changed twice a week. Cells were used for experimentation or split when 80–90% confluent.

The human hepatoma cell line HepG2 were maintained in Eagle's minimal essential medium (EMEM) (Sigma-Aldrich, UK) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, UK), 1% penicillin–streptomycin (Sigma-Aldrich, UK), 1% glutamine (Gibco, UK) and 1% non-essential amino acids (Sigma-Aldrich, UK). Cells were kept at 37 °C under a humidified atmosphere containing 5% CO₂. The medium was changed twice a week. Cells were used for experimentation or split when 80–90% confluent.

2.2.2. Cell Viability Assay

Cell viability assay is a colorimetric method that is based on the reduction of 3-[4,5 dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases, into insoluble purple formazan crystals. The crystals are then dissolved in dimethyl sulfoxide (DMSO) and analysed spectrophotometrically. Approximately, 10⁴ cells per

well were seeded into 96-well plates using Minimum Essential Medium Eagle (MEM). The cells were left to adhere overnight before proceeding with various treatments. After the treatment, cells were washed with PBS, 90 μ L of Dulbecco's Modified Eagle Medium (DMEM) and 10 μ L of MTT solution [5 mg of MTT diluted per mL of Phosphate-Buffered Saline (PBS)] were added to each well and incubated at 37 °C for 3 hours. Next, the formazan crystals were dissolved in DMSO, and the absorbance was read at 570 nm and 650 nm by BioTek Synergy LX plate reader. The cell viability is equivalent to the formation of the formazan crystals and is expressed as a percentage of the control values (2).

2.2.3. Fenton substrate challenge

To investigate the protective effects of polyphenols against iron-induced oxidative stress, cells were exposed to a Fenton substrate consisting of 30 μ M of FeSO_4 and 0.5 mM of H_2O_2 for 2 h (3). Briefly, cells were pre-supplemented with different concentrations of polyphenols and incubated at 37 °C overnight. The cells were then exposed to a Fenton substrate for 2 hours, followed by a cell viability analysis or other functional assays. Control cells were treated only with the cell culture medium (MEM).

2.2.4. Iron-Induced Stress

Cells were pre-treated with polyphenols for 24 h before exposure to rapid iron overload damage (4) with 50 μ M FAC and 20 μ M 8HQ (8HQ-FAC). Cells were incubated for a further 2 h at 37 °C to allow rapid iron uptake. Afterwards, cell viability was determined with the MTT assay and other functional assays were performed. Control cells were treated only with the cell culture medium (MEM).

2.2.5. Ferroptotic Activity in Cells

Cells were treated ferroptosis inducer, erastin at certain amounts to show ferroptotic cell death. Cells were incubated for a 24 h at 37 °C to allow rapid ferroptotic cell damage. To show the protective effects of several polyphenols, cells were co-treated with polyphenols and erastin for 24 h, and incubated at 37 °C. Afterwards, cell viability was determined with the MTT assay and other functional assays were performed. Control cells were treated only with the cell culture medium (MEM).

2.2.6. Measurement of intracellular reactive oxygen species

Reactive oxygen species was monitored using dihydrodichlorofluorescein (H_2DCF) cell-permeant probe according to the manufacturer's recommendations. Briefly, MIN6 cells from

different groups were collected and washed with PBS and then incubated for 90 minutes in the dark at 37 °C. in PBS containing 10 μ M of H₂DCF. The level of ROS generated was measured using flow cytometry based upon the fluorescence intensity of DCF at 525 nm after excitation at 485 nm. The levels of ROS were expressed as units of fluorescence compared with that of control group.

2.2.7. Chelation Assay

Ferrous iron chelating potentials or capacity of polyphenols including ferulic acid and ferulic acid 4-sulfate, compared with DFO were determined with a ferrozine assay as described (5). Briefly, different concentrations (2.5, 5, 10, 20, 40, 80 μ l) of DFO were mixed with 30 μ M of FeSO₄ and left to stand for 30 min at room temperature. Ferrozine (75 μ M) was added to the mixture and left for a further 30 minutes at room temperature. After incubation, absorbance was measured at 562 nm by BioTek Synergy LX plate reader. The control was FeSO₄ plus ferrozine and FeSO₄ plus FA, FAS and DFO was used as a blank. The ability of FA, FAS or DFO to chelate iron was calculated using the equation below:

$$\text{Iron chelating ability (\%)} = (\text{control} - (\text{treatment} - \text{blank})) / (\text{control})$$

2.2.8. Lipid peroxidation assay (MDA) in cells

The assay was performed using the malondialdehyde microplate assay kit from Cohesion Biosciences (London, UK). Malondialdehyde (MDA) is the endpoint product of lipid peroxidation and an oxidative stress marker. Approximately, 10⁶ cells were seeded in 6-well plates with MEM until confluency was achieved. Cells were pre-treated or co-treated with polyphenols and treated with iron substrates or ferroptosis inducers for 24 hours to induce iron-induced cell damage. Control cells were treated only with MEM. After the incubation, cells were washed with cold PBS, detached from the wells by scratching and cells from two wells were collected into one Eppendorf tube. After centrifuging the samples at 12.000 g for 5 minutes at 40 °C, the cell pellet was mixed with assay buffer to lyse the cells by vortex and sonication for 15 minutes at the 100-power setting, a second centrifugation at 12.500 g for 10 minutes at 40 °C was performed. The assay starts with 100 μ L of the supernatant mixed with 200 μ L dye reagent containing thiobarbituric acid (TBA) and incubated for 30 minutes at 90 °C. The samples were centrifuged for 10 minutes at room temperature and the absorbance of the supernatant was read at 532 nm and 600 nm. The protein concentration of the extracts

was analysed with the Bradford assay protocol and the MDA values were normalized against protein content.

2.2.9. Total Protein content

The protein content of the cells/tissue was determined using the Bio-Rad DC protein assay Kit. The assay is based on the reaction of protein with the alkaline copper tartrate solution and Folin reagent. The kit consisted of reagent A (alkaline copper tartrate solution), reagent B (dilute Folin Reagent), and reagent S. A working reagent was prepared by mixing reagents S and A together (50:1). 20 µl of the disrupted cell were added to 20 µl of the working reagent (solutions S and A) to this mixture 160 µl of solution B was added, mixed, and left to incubate at room temperature for 15 minutes, afterwards absorbance was read at 750 nm on a spectrophotometric BioTek Synergy LX plate reader. The protein assay solution was made in duplicates in a 96 well plate. Standards of bovine serum albumin (BSA) were dissolved in Hank's Balanced Salt Solution were run (Appendix II). The absorbance of the samples was calculated against the intercept of the standard curve.

2.2.10. Total glutathione (TGS) assay

The method as described by Akerboom and Sies (6) is a kinetic assay in which the total content of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured by the continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by the dihydronicotinamide adenine dinucleotide phosphate (NADPH) (6). The assay was performed by using the Glutathione Assay kit by Sigma-Aldrich (Dorset, UK). Cells were seeded in 6-well plates with MEM until 80% confluency was achieved. Then, cells were pre-treated or co-treated with polyphenols and treated with iron substrates or ferroptosis inducers to induce iron damage for 24 hours. After the incubation, cells were washed with ice-cold PBS, and detached, collected into Eppendorf tubes, centrifuged at 600 g for 10 minutes at 40 °C to obtain a thick pellet and the supernatant was discarded. After the pellet was vortexed, its volume was measured and 3 volumes of 5% Sulfosalicylic Acid (SSA) were added to each sample. Freeze and warm cycles were performed twice using liquid nitrogen for snap-freezing the samples and a 37 °C water bath to thaw them. Finally, the samples were centrifuged at 10,000 g for 10 minutes at 40 °C. The supernatant was collected and mixed and incubated for 5 minutes with the working mixture containing; 100 mM potassium phosphate buffer and 1 mM EDTA, pH 7.0 (Assay buffer), an enzyme solution (228 µL) of glutathione reductase (6

units/mL) and 228 μ L of DTNB solution (1.5 mg/mL). The mixture was boiled for 30 minutes, and 100 μ L of the final product was added to a 96-well plate. The absorbance was read at 412 nm at 1-minute intervals for 5 minutes when the NADPH solution (0.16 mg/mL, 2.5 mL assay buffer) was added to each well. Tecan Sunrise microplate reader and Magellan software were used to read at what wavelength the absorbance. A standard curve of known concentrations of reduced glutathione was measured every time along with the samples.

2.2.11. CuZn-Superoxide dismutase (SOD) Analysis

Superoxide dismutase activity was measured using the Amplite™ Colorimetric Superoxide Dismutase (SOD) Assay Kit (Cat. 11305). The protocol was performed according to the manufacturer's instructions. The assay is based on the reaction of superoxide with SOD Orange™ to generate a product that absorbs light around 560 nm. SOD inhibits the reaction of SOD Orange™ in the presence of superoxide, thus reducing the absorption at 560 nm. The reduction in the absorption of SOD Orange™ at 560 nm is proportional to SOD activity. Samples were homogenized in ice-cold PBS with a glass homogenizer, centrifuged at 10,000 $\times$ g for 15 minutes at 4 °C. The supernatant was collected and diluted at 1:50 in the assay buffer. Standard samples of mouse superoxide dismutase provided in the kit were freshly reconstituted in assay buffer within 10 minutes before the reaction. The colorimetric reaction started by adding 50 μ L of experimental samples, standard SOD, or assay buffer as a blank, in a 96 well plate, followed by the addition of 50 μ L of the working solution. Afterwards, the plate was incubated at room temperature for 45 minutes and the absorbance was read at 560 nm by BioTek Synergy LX plate reader.

2.2.12. Cellular Iron Levels

Inductively-coupled plasma mass spectrometry (ICP-MS) analysis of total cellular iron level was performed. Cell pellets collected for metal analysis by ICP MS were re-suspended in 200 μ L of 50 mM NaOH. Concentrated 68% HNO₃ (nitric acid) was added to the samples and the samples were then heated for 3 h at 80 °C to complete the digestion. Measurements were made using an Agilent ICPMS 7700 x series ICP MS instrument under operating conditions suitable for routine multi-element analysis.

2.2.13. Glucose-stimulated insulin secretion assay

Cells were seeded in 96-well plates at a density of 4×10^4 cells per well. After treatment with FAC, the cells were then starved overnight in low glucose DMEM containing 15% FBS and 2

mmol/L glucose. On the day of experiment, cells were washed twice and incubated for 1 hour in 100 μ L Krebs Ringer Bicarbonate Buffer (KRBB) (140 mmol/L NaCl, 3.6 mmol/L KCl, 0.5 mmol/L NaH_2PO_4 , 0.2 mmol/L MgSO_4 , 1.5 mmol/L CaCl_2 , 10 mmol/L HEPES, 2 mmol/L NaHCO_3 and 0.5% bovine serum albumin) containing 2 mmol/L glucose. Following this, KRBB containing 20 mmol/L glucose and FAC was applied to cells. To assure the validity of the assay, a positive control treated with 20 mmol/L of KCl and a negative control treated with 2 mmol/L glucose were used. Cells were incubated for 3 hours to allow glucose-stimulated insulin secretion. Insulin levels in the buffer were determined using an anti-rat/mouse insulin ELISA kit (Millipore; EZRMI-13K) with mouse or rat insulin as a standard in accordance with the manufacturer's instruction. The absorbance was read at 450 nm using a microplate reader (BioTek Synergy LX plate reader).

2.2.14. Western Blot

Cells were lysed with RIPA Buffer (Tris/Cl (pH 7.6); 100 mmol/L, EDTA; 5 mmol/L, NaCl; 50 mmol/L, β -glycerophosphate; 50 mmol/L, NaF; 50 mmol/L, Na_3VO_4 ; 0.1 mmol/L, NP-40; Sodium deoxycholate; 0.5%), supplemented with Protease Inhibitor Cocktails (Thermo Scientific, Dartford, UK). Protein concentration was determined using Bio-Rad reagents (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms (20 μ g) of protein extracts were loaded onto a 12% gel in SDS polyacrylamide gels (SDS-PAGE). The resolved proteins were transferred onto PVDF membranes and blocked with 5% non-fat dry milk in TBST buffer (20 mM Tris-Base, pH 7.4, 500 mM NaCl, 0.1% Tween20). The membranes were probed with a primary antibody and a secondary antibody diluted in TBST and incubated overnight at 4 °C. Afterwards, the membranes were probed with HRP-conjugated secondary antibody (diluted 1:5000, R&D Systems, Abingdon, UK) for 1 h at room temperature. Cross-reactivity was observed with peroxidase-linked anti-IgG by using Clarity Western ECL Substrate (Watford, UK). Densitometry of the band intensities was determined using Image J software (National Institute of Health, USA).

2.2.15. Study design of animal experiment

Thirty-five BALB/c male mice, 3 weeks old and 24-30 g each were housed at 21-23 °C in a 12-hour dark/light cycle. The animals were nourished with a standard laboratory pellet diet and water ad libitum. Animal care and all procedures were conducted in accordance with methods approved by the College Research Ethics Committee of King's College London, and the United

Kingdom Animals (Scientific Procedures) Act 1986 (ASPA). Animals were divided randomly into seven groups (n=5), (1) Control, (2) Iron dextran (ID), (3) FA, and (4) FAS, (5) dimethyl sulfoxide (DMSO), (6) FA + iron dextran, (7) FAS+ iron dextran. The iron dextran group received five doses of iron dextran injected intraperitoneally (i.p.) every two days 100 mg/per kg mice for 10 days to induce the iron overload condition. Mice were given FA and/or FAS, which was dissolved in 0.1% DMSO, for 10 days by gavage, starting a day before the commencement of iron loading (100 μ L of 20 mg/per kg of FA or FAS). The control untreated mice were given sterile saline by gavage for 10 days. Another group was administered with 0.1% DMSO. Euthanasia of the mice was performed on the day 10 by injecting 0.4–0.6 mL of pentobarbitone sodium 20% w/v solution (i.p.) to the mice. Tissues of liver, spleen, and kidney were collected and flash-frozen with liquid nitrogen and kept at -70 °C. In addition, samples of tissues from the mice were embedded in optimal cutting temperature (OCT) compound for histochemical analysis.

2.2.16. Non-haem iron determination in mice tissues

Non-haem iron was evaluated according to the modified method of Simpson and Peters (7). Briefly, protein precipitation is performed with trichloroacetic acid (TCA). Ferric iron is released with sodium acetate solution (pH 4.8), which is reduced to ferrous iron Fe (II) by sodium ascorbate which then reacts with sodium 3-(2- pyridyl)-5,6-bis (4 phenylsulfonate)-1,2,4-triazine (ferrozine) to form a purple Fe-ferrozine complex that can be read spectrophotometrically (7).

Tissue samples of liver, kidney, and spleen were washed with ice-cold 0.15 M NaCl to remove any excess blood. Known weights of the samples were then homogenized in 500 μ L of 0.15 M NaCl in 10 mM NaOH-HEPES buffer (pH 7.4) in a 1 mL glass Dounce homogenizer. In a screw cap tube, 100 μ L of the homogenate was mixed with 200 μ L of extraction buffer mixture (25% TCA in 4% sodium pyrophosphate). The tube was placed in a boiling water bath for 10 minutes, centrifuged for 5 minutes at 14,500 g and the supernatant was collected in a new Eppendorf tube. The precipitate was re-suspended with 100 μ L of TCA, heated and further centrifuged. After a third extraction, 200 μ L of the overall extract was mixed with 100 μ L of 0.23 M ascorbic acid, 80 μ L of 0.01 M ferrozine and 420 μ L of 2.0 M sodium acetate buffer (pH 4.8). The blank and the standard solutions were prepared similarly except that 200 μ L of TCA was added to each tube. As a standard, 1.0 μ L of 10 mM ferric chloride [FeCl₃ dissolved

in 10 mM hydrochloric acid (HCl)] was used. The optical densities of all tubes were measured at 562 nm against the blank using a Camspec M330 UV-Visible spectrophotometer.

2.2.17. Tissue iron staining

Iron staining by Perl's protocol is a common method that is widely used to visualize ferric iron molecules of a specimen (8). In short, the insoluble Prussian blue compound is formed when sections are treated with acidic potassium ferrocyanide. The acid releases non-haem iron molecules from the tissues which then react with the ferrocyanide ion to form a water-insoluble compound of blue colour (Prussian blue). More specifically, tissues were cut into 7 µm thick sections at -20 °C and placed on poly-L-lysine coated slides. The sections were left to air dry for one hour at room temperature and fixed with 50% methanol and 50% acetone solution for 10 minutes and washed with PBS for 5 minutes. Sections were incubated at 65 °C for 2 hours with equal volumes of 5% of acetic acid and 2% potassium ferrocyanide and rinsed with PBS. Neutral Fast Red solution was used to counterstain the sections for 5 minutes. Afterwards, sections were rinsed a final time with PBS and mounted using FluorSave reagent. All glassware equipment was washed before use with 10% HCl and 10% nitric acid solution overnight and the use of metallic forceps was avoided. Images were taken using Nikon Eclipse TE200 light microscope at 10x magnification, captured using the Nikon DXM1200 digital camera and processed with Lucia G xv4.61 software. Sections that were not treated with potassium ferrocyanide served as negative controls. Two slides per group were analysed.

2.2.18. Tissue malondialdehyde assay (MDA) analysis

Mice tissues were pulverized by grinding in a mortar with liquid nitrogen. A known weight of tissue was homogenized in 1 mL glass Dounce homogenizer using ten times the volume of the assay buffer provided to lyse the tissue. The assay was performed as in section 2.2.8.

2.2.19. Tissue total glutathione (TGSH) analysis

A known weight of mice tissue was pulverized with liquid nitrogen to create a fine powder. The powder was homogenized in 1 mL glass Dounce homogenizer with 10 volumes of cold 5% Sulfosalicylic Acid (SSA) to deproteinize the sample. After 10 minutes of incubation on ice, the samples were centrifuged at 10,000 g for 10 minutes at 40 °C and the supernatant was used for the determination of total glutathione as previously described for the assay with cells (Section 2.2.10).

2.2.20. Tissue CuZn-Superoxide dismutase (SOD) Analysis

A known weight of tissue from the animal experiments was pulverized with liquid nitrogen to create a fine powder and was then homogenized with assay buffer in 1 mL glass Dounce homogenizer to lyse the tissues. The colorimetric reaction started by adding 50 μ L of experimental samples, standard SOD, or assay buffer as a blank, in a 96 well plate, followed by the addition of 50 μ L of the working solution. Afterwards, the plate was incubated at room temperature for 45 minutes and the absorbance was read at 560 nm (Section 2.2.11).

2.2.21. Western blot analysis of tissues

Total proteins were extracted from liver and pancreatic tissues by homogenization in Radio Immunoprecipitation Assay (RIPA) lysis buffer containing 20 mM KH_2PO_4 , 0.1 mM EDTA, 135 mM KCL, supplemented with Protease Inhibitor Cocktails (Thermo Scientific, Dartford, UK). Protein concentration was determined using Bio-Rad reagents (BioRad, Munich, Germany) and 20 μ g protein of each sample was mixed with loading buffer, denatured at 95 °C for 5 min, and separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The resolved proteins were transferred onto PVDF membranes and blocked with 5% non-fat dry milk in TBST buffer (20 mM Tris–Base, pH 7.4, 500 mM NaCl, 0.1% Tween20) for 1 h at room temperature. The membranes were probed with a primary antibody diluted in TBST and incubated overnight at 4 °C. Afterwards, the membranes were probed with HRP-conjugated secondary antibody (diluted 1:5000, R&D Systems, Abingdon, UK) for 1h at room temperature. Cross-reactivity was observed with peroxidase-linked anti-IgG by using Clarity Western ECL Substrate (Watford, UK). The images were quantified using ImageJ software (NIH, Bethesda, MD, U.S.A.) and normalized to respective loading controls.

2.2.22. RNA extraction

RNA was extracted from the tissues using Trizol (Cat. No. 15596026, Thermofisher, Scientific) RNA isolation protocol. Sample 100 mg of each tissue was homogenized in 800 μ L of Trizol reagent using a glass homogenizer. The resulting product was incubated for 5 minutes at room temperature and afterwards centrifugated at 10,000 x g for 5 min at 40 °C to remove cell debris. The supernatant was transferred into a new Eppendorf tube. After centrifugation, 0.2 ml of chloroform was added and again centrifuged at 12,000 x g for 15 minutes at 40 °C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, interphase, and a colourless upper aqueous phase. The upper aqueous phase, containing the

RNA, was carefully transferred to a new 1.5 ml microfuge tube without disturbing the interphase. Finally, to precipitate the RNA, 0.5 ml of isopropyl alcohol was mixed with the aqueous RNA phase and incubated at room temperature for 10 minutes. Further centrifugation was performed at 10,000 x g for 10 minutes at 40 °C and the supernatant was removed. The RNA pellet was washed with 75% ice-cold ethanol, centrifuged at 7,500 x g for 5 minutes, and washed again, in total was washed three times. After washing, the remaining pellet after the last centrifugation was air-dried for 10 minutes. Final samples were dissolved in 200 µl of pure water or RNase free H₂O and absorbance was read in a Nanodrop spectrophotometer at 260 nm to estimate RNA concentration. RNA purity was judged or checked by A260/A280 ratios of the samples.

2.2.23. cDNA synthesis

cDNA was synthesized by reverse transcription from the RNA using the Transcriptor High Fidelity cDNA Kit (Roche Diagnostic). The first cDNA strand was prepared with 1 µg of RNA dissolved in RNase free H₂O and 0.5 µL of oligo DT was added to the mixture. Denaturation was done at 65 °C for 10 minutes in a thermal cycler. The master mix was prepared as shown in Table 2.1. Reaction mixtures were incubated at 25 °C for 10 minutes in a thermal cycler at 37 °C for 120 min and the synthesis was terminated at 85 °C for 5 minutes, letting the samples to cool at 40 °C.

Table 2.2. Master mix preparation for cDNA synthesis.

Constituent	Volume/Reaction (µL)
10× RT Buffer	2
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2
MultiScribe™ Reverse Transcriptase	1
RNA sample	(1 µg)
water	To complete the total volume
Total per Reaction	10

2.2.24. Real-time polymerase chain reaction (RT-qPCR)

RT-qPCR was conducted in the ABI Prism, 7900 HT Fast RT-qPCR (Applied Biosystems). The cDNA sample and the master mix (Table 2.2) were aliquoted from a 96 to a 376 well plate with the Biomek FX liquid handling robot, Beckman Coulter. After aliquoting, the samples were placed in the ABI Prism equipment qPCR reactions were performed at the following conditions:

40 cycles	Denaturation	50 °C	2 minutes
	Annealing	95 °C	10 minutes
	Amplification	95 °C	15 sec
	Extension	60 °C	1 minute

Table 2.3. Preparation of Master mix for real time qPCR.

Constituent	Final Concentration	µl per reaction
TaqMan master mix	1x	5
F primer	0.2 µM	0.2
R primer	0.2 µM	0.2
UPL	0.1 µM	0.1
water		0.5
cDNA	20 ng	4
Total		10

Table 2.3 shows the primers used for the amplification of iron gene transporters and antioxidant markers. The cycle threshold (Ct) was obtained at the end of the qPCR reaction. The gene expression fold changes were calculated using $\Delta\Delta C_t$ method by Livak and

Schmittgen (9). The amplified genes were normalised with ribosomal protein L19 (RPL19) as the reference gene.

Table 2.4. Primers sequences used for qPCR.

Genes	Forward primer	Reverse primer
NFR2	catgatggacttgagttgc	cctccaaaggatgtcaatcaa
GPx4	tttctgacacaggttcact	cagcctggtctgtaagca
HO-1	agggtcaggtgtccagagaa	cttcagggccgtgtagata
NQO1	agcgttcggtattacgatcc	agtacaatcagggtcttctcg
GCLC	agatgatagaacacgggaggag	tgatcctaaagcgattgttcttc
STAT3	gttcctggcaccttgatt	caacgtggcatgtgactctt
SOCS3	atttcgcttcgggactagc	aacttgctgtgggtgacat
Foxo1	cttcaaggataaggcgaca	gacagattgtggcgaattga
Trf1	catgagggaatcaatgatcg	ctaaagctgagagagtgtagagc
DMT1	ggctcctgggatatggagtc	tctgtgctcttagaataggattcg
FPN	gggtttcttagaagcaggtatgc	ttctcagtgtagacacattcaagtc
FtH	ggacacggtgatgagagctaa	aaaagatgaaggcagcctga
FtL	cagccgcctttacaagtctc	taggagctaaccggaagag
RPL19	ccacaagctctttcctttcg	ggatccaaccagaccttctt

2.2.25. Statistical Analysis

Statistical analysis was performed by GraphPad Prism 8.0 (GraphPad Software, USA) using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-hoc test to compare the means of the experimental groups. All the values are expressed as mean $\pm$ SEM. The test was considered significant when a *p* value was less than 0.05.

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Chapter 3. Curcumin and (–)- Epigallocatechin-3-Gallate Protect Murine MIN6 Pancreatic Beta-cells Against Iron Toxicity and Erastin-Induced Ferroptosis

Abstract

Ferroptosis is a form of programmed cell death that is characterized by lipid peroxidation and is inducible by iron and the accumulation of reactive oxygen species (ROS). It is triggered by erastin but inhibited by antioxidants such as α -tocopherol, β -carotene, polyphenols, and iron chelators such as deferoxamine (DFO), nitrilotriacetic acid (NTA), and ethylenediaminetetraacetic acid (EDTA). This study investigated the protective effects of two polyphenols, curcumin and (–)- epigallocatechin-3-gallate (EGCG), against iron loading and erastin-mediated ferroptosis in MIN6 cells. Cells were treated with 20 μ M polyphenols before exposure to iron-induced oxidative stress comprising of 20 μ M of 8-hydroxyquinoline (8HQ) and 50 μ M of ferric ammonium citrate, (FAC) (8HQ+FAC) or Fenton reaction substrate (FS) (30 μ M of FeSO_4 and 0.5 of mM H_2O_2) and 20 μ M erastin. Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay, iron levels were measured by inductively-coupled plasma mass spectrometry (ICP-MS), glutathione and lipid peroxidation were assayed with commercially-available kits. Curcumin and EGCG both significantly protected pancreatic cells against iron-induced oxidative damage. Moreover, both compounds also protected against erastin-induced ferroptosis in pancreatic cells. The polyphenols enhanced cell viability in erastin-treated MIN6 cells in a dose- and time-dependent manner. Furthermore, MIN6 cells exposed to erastin alone showed elevated levels of iron, glutathione (GSH) depletion, glutathione peroxidase 4 (GPX4) degradation and lipid peroxidation ($p < 0.05$) compared to cells that were protected by pre-treatment with curcumin or EGCG. Taken together, the data identify curcumin and EGCG as novel ferroptosis inhibitors, which might exert their protective effects by acting as iron chelators and preventing GSH depletion, GPX4 inactivation, and lipid peroxidation in MIN6 cells. The implications of the findings on the effects of iron overload and ferroptosis represent a potential therapeutic strategy against iron-related diseases.

3.1. Introduction

Iron is a vital trace metal in physiological processes such as oxygen transport, respiration, energy generation and DNA synthesis, and is a structural component of numerous enzymes and proteins in the body. The ability of iron to perform these functions relies on its existence in variable and interconvertible oxidation states. Iron can exchange single electrons with metabolites to generate reactive oxygen species (ROS) that are capable of causing DNA damage, protein denaturation, and lipid peroxidation (1). Together, these events have recently been defined as a form of programmed cell death called ferroptosis (2). Excessive ROS production is neutralized by a repertoire of defence antioxidants enzymes such as superoxide dismutase, catalase, and glutathione peroxidases (GPXs) (3). Dysregulation of iron homeostasis can lead to iron-overload disorders such as hemochromatosis and thalassemia, which are characterized by elevated levels of ROS. It is noteworthy that there is a high prevalence of type 2 diabetes mellitus (T2D) amongst patients (4), and epidemiological studies have reported significant associations between excess iron stores and T2D (5,6). Toxicity of iron in pancreatic β -cells, possibly because of elevated ROS, results in decreased insulin synthesis and secretion (6). Iron overload-mediated oxidative stress causes impaired insulin signaling and inhibition of ATP production due to mitochondrial dysfunction (6,7). Furthermore, reduced antioxidant levels in conjunction with increased free radical production have been reported to cause pancreatic β -cell damage (8,9).

Although synthetic iron chelators such as deferoxamine (DFO), nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA) are used in treating secondary iron overload disorders, in particular natural plant products or bioactive compounds of plant origin such as polyphenols are emerging as potent and efficacious alternatives (10,11). Moreover, in addition to their iron chelating properties, some polyphenols also possess antioxidant potential. Polyphenols such as curcumin and (–)- epigallocatechin-3-gallate (EGCG) have been shown to form complexes readily with different metal ions (12,13).

Ferroptosis emerged recently as a phenomenon that encapsulates the interplay of iron accumulation, excessive ROS levels and lipid peroxidation, and culminates in cell death that is associated with degenerative disorders (14). In light of this, the current study investigated the protective functions of polyphenols against iron toxicity and erastin-induced ferroptosis

in murine MIN6 cells. In essence, the investigation was designed to demonstrate the inhibition of erastin-induced ferroptosis, iron accumulation, and lipid peroxidation by curcumin and EGCG inclusion in treated MIN6 pancreatic cells.

3.2. Results

Protective Effects of Polyphenols on Iron-Induced Oxidative Stress

Initial experiments investigated the sensitivity of MIN6 pancreatic cells to iron-induced stress and the protective effects of polyphenolic compounds. Based on in vitro studies, six different polyphenols including curcumin, quercetin, rutin, EGCG, tannic acid or phytic acid were decided to examine iron related cell damage at 20 μ M concentration. In cells pre-incubated with six different polyphenols before exposure to FS and 8HQ+FAC for 2 h, only curcumin and EGCG showed significant protection against iron-induced cell damage (Figure 3.1A and B).

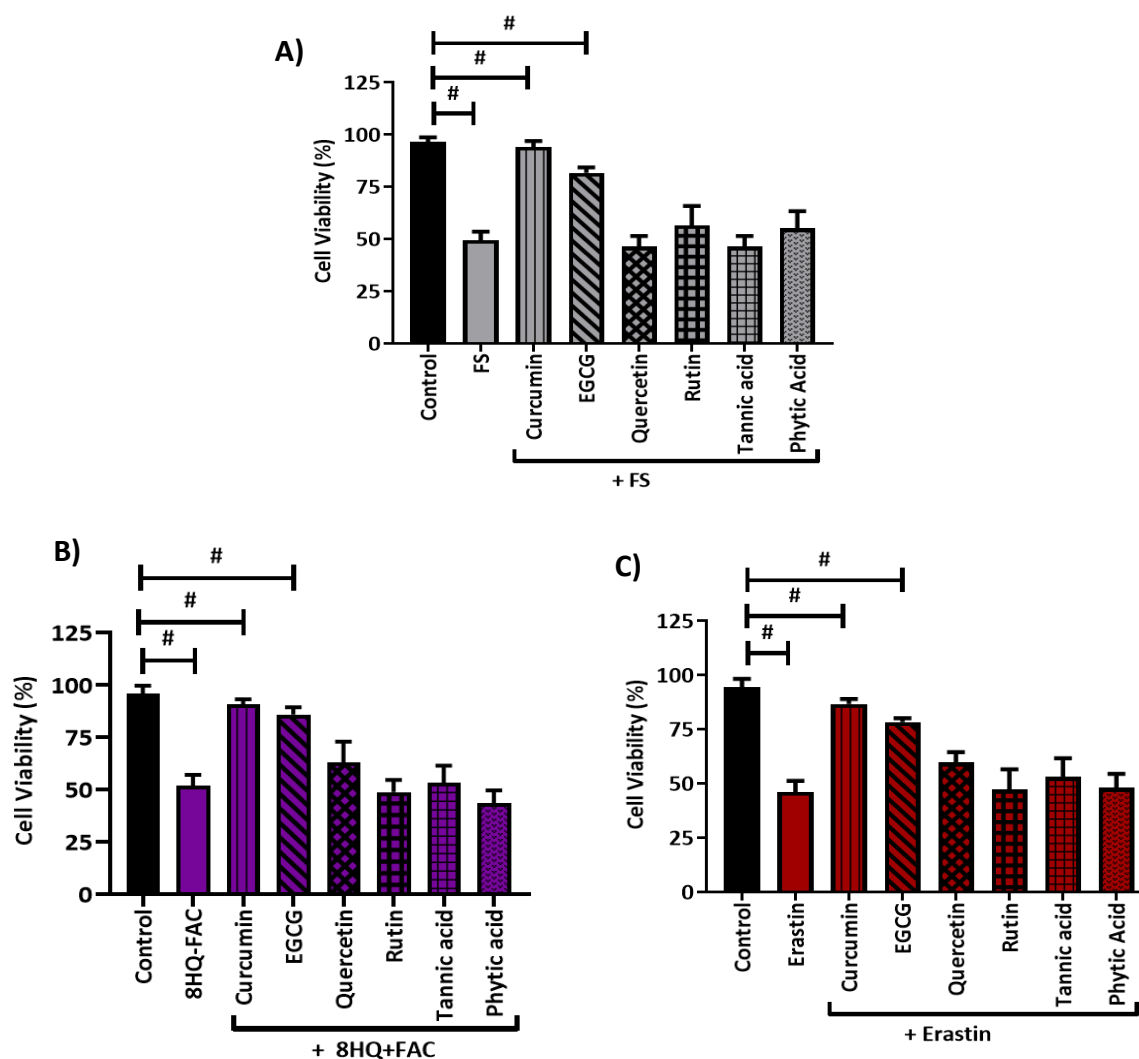


Figure 3.1. Protective effects of different polyphenols against iron-mediated toxicity and ferroptosis.

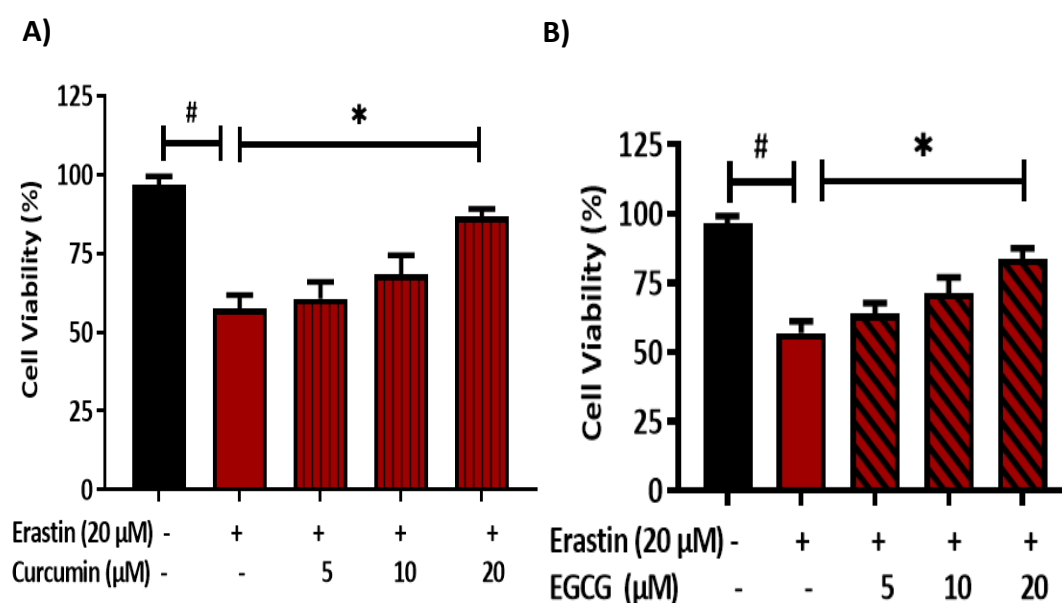
MIN6 cells were pretreated with 20 μ M curcumin, quercetin, rutin, EGCG, tannic acid or phytic acid for 24 h and, then supplemented with (A) Fenton Substrates (FS), (B) 8HQ+FAC for 2 h and (C) MIN6 cells were treated overnight with 20 μ M erastin, in the absence or presence of 20 μ M quercetin, rutin, curcumin, tannic acid, phytic acid and EGCG. The percentage of cell viability is relative to control cell samples. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control vs. treatment groups, * $p < 0.05$ compared with FS, 8HQ+FAC or erastin group only.

Protective Function of Curcumin and EGCG against Ferroptosis

Having identified curcumin and EGCG as the most potent polyphenols for conferring protection against iron-induced stress, we next investigated the potential of these compounds to act as inhibitors of ferroptosis. Subsequently curcumin and EGCG were compared with quercetin, rutin, tannic acid and phytic acid as inhibitors of erastin-induced ferroptosis in MIN6 cells. Of the six compounds that were tested, curcumin and EGCG exhibited the best protection against erastin-induced cell death in MIN6 cells (Figure 3.1C).

Dose-Response Effects of Curcumin and EGCG against Erastin-Induced Ferroptosis

Cell viability was evaluated in cells exposed to a range of EGCG and curcumin concentrations in the presence of erastin for 24 h. Curcumin caused a decrease in cell mortality in a dose-dependent manner, over a concentration range of 5–20 μ M in MIN6 cells (Figure 3.2A). Likewise, EGCG also inhibited erastin-induced cell death in a dose-dependent manner in MIN6 cells (Figure 3.2B).



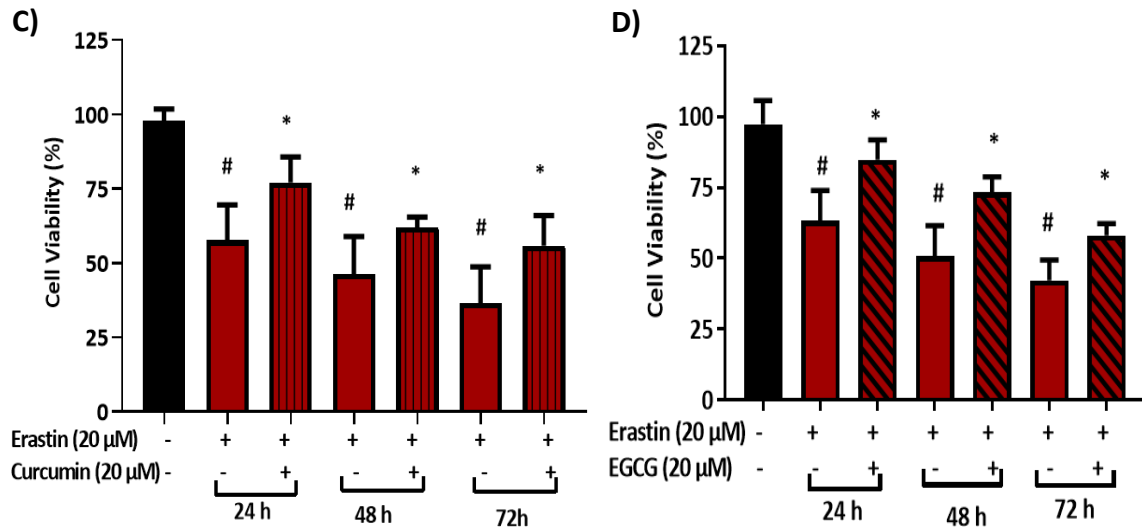


Figure 3.2. Anti-ferroptosis activity of curcumin and EGCG in MIN6 cells. MIN6 cells were treated overnight with 20 μ M erastin in the absence or presence of curcumin (A) or EGCG (B) in a dose-dependent manner. MIN6 cells were treated overnight with 20 μ M erastin in the absence or presence of 20 μ M curcumin (C) or EGCG (D) in a time-dependent manner. All the values are expressed with the mean $\pm$ SEM, $n = 8$, Experiments were repeated 3 independent times. [#] $p < 0.05$ control vs. treatment groups, ^{*} $p < 0.05$ vs. erastin only. One-way ANOVA, Tukey post-hoc test.

Time Course Effects of Curcumin and EGCG against Erastin-Induced Ferroptosis

Next, we determined the temporal range of protection conferred by curcumin or EGCG against erastin-induced ferroptosis. Protection was evident with both curcumin and EGCG in MIN6 cells after 24 h. Over these specific time points, both polyphenols provided a similar pattern of protection against erastin-induced cell death in MIN6 cells (Figure 3.2C and D).

Curcumin and EGCG Limit Iron Accumulation and Lipid Peroxidation in Ferroptosis

As iron accumulation is a known cause of ferroptotic cell death (14), we next investigated whether curcumin and EGCG would suppress iron accumulation resulting from erastin treatment in MIN6 cells. Intracellular iron level in MIN6 cells following exposure to erastin was almost 2 fold greater than in untreated control cells (Figure 3.3A). Treatment with curcumin or EGCG resulted in a decrease in erastin-induced iron accumulation in MIN6 cells. In parallel, the effect of erastin on the lipid peroxidation marker malondialdehyde (MDA) was measured. MDA levels were increased significantly following treatment with erastin, but were suppressed by co-exposure to curcumin and EGCG (Figure 3.3B) ($p < 0.05$).

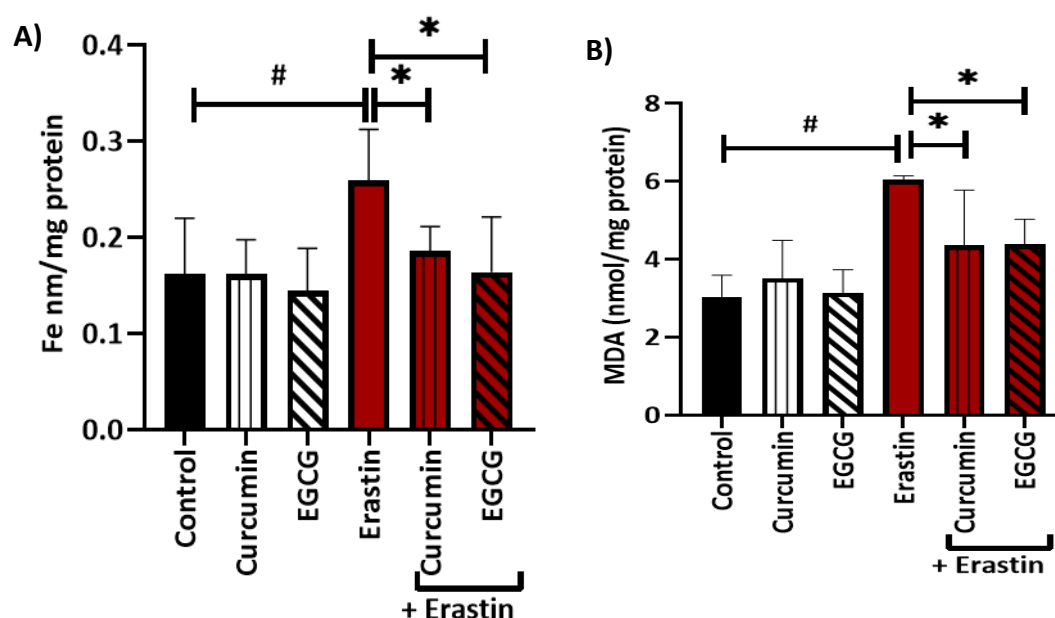


Figure 3.3. Curcumin and EGCG suppress iron and lipid accumulation in pancreatic cells. Cells were cotreated overnight with 20 μ M erastin in the absence or presence of curcumin or EGCG. (A) Amount of Fe is relative to control cell samples. (B) Level of MDA is relative to control cell samples. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control vs. treatment groups, * $p < 0.05$ vs. erastin only. One-way ANOVA, Tukey post-hoc test.

Curcumin and EGCG Prevent Glutathione (GSH) Depletion and Glutathione Peroxidase 4 (GPX4) Degradation

GSH level was reduced in MIN6 cells exposed to erastin and co-treatment of cells with curcumin or EGCG significantly abrogated GSH decline (Figure 3.4A). Moreover, treatment of MIN6 cells with erastin also resulted in decreased GPX4 protein levels (Figure 3.4B,C), and curcumin significantly inhibited this response in the cells ($p < 0.05$).

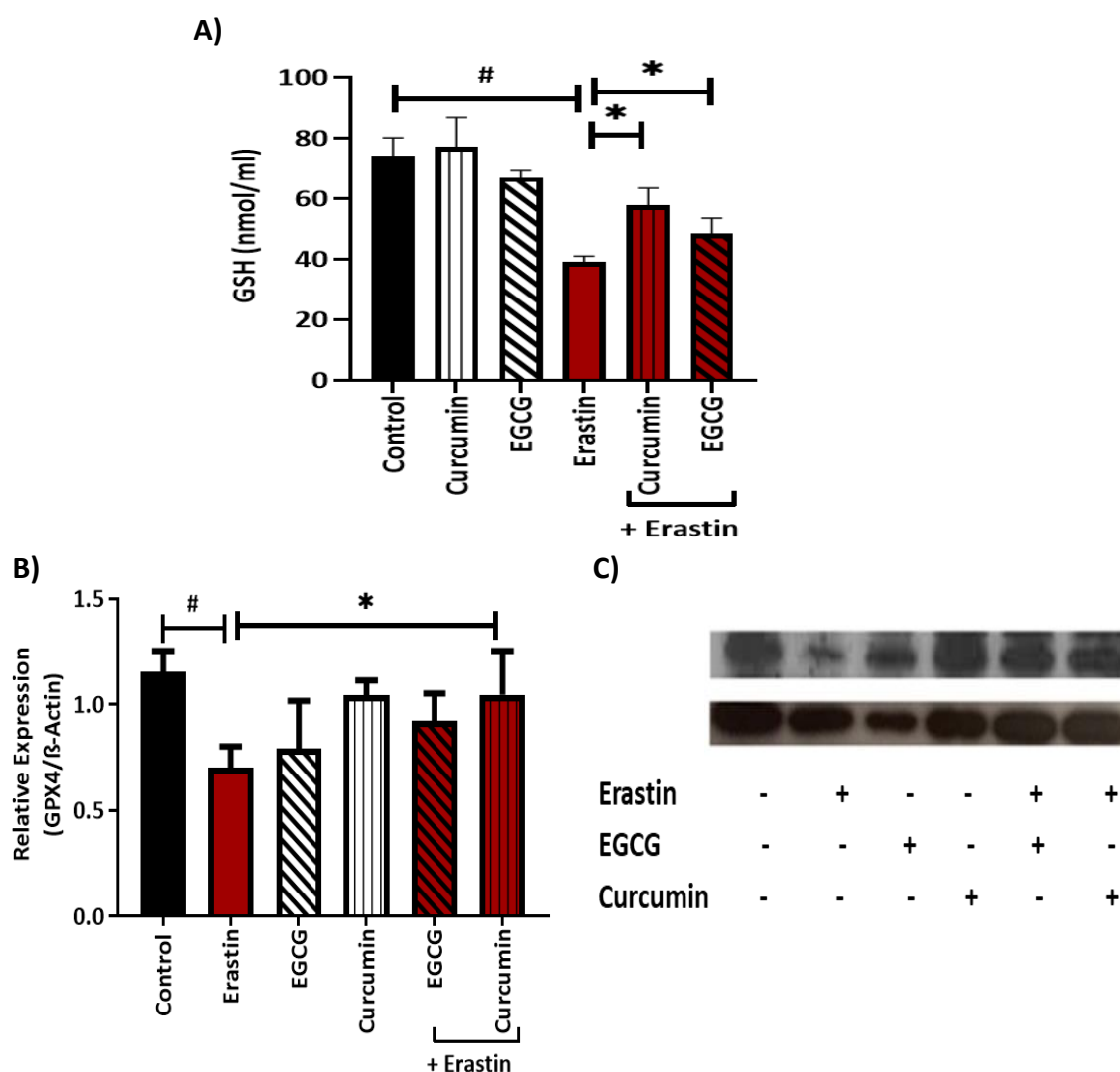


Figure 3.4. Curcumin and EGCG inhibit GSH depletion in pancreatic cells. MIN6 cells were cotreated overnight with 20 μ M erastin in the absence or presence of curcumin or EGCG. GSH levels were measured in the cell samples (A). Total cell lysates were prepared and subjected to Western blot analysis to monitor the expression levels of antioxidant protein of GPX4 (B). Densitometry of Western blots GPX4 protein bands of GPX4 (C). All the values are expressed with the mean $\pm$ SEM, $n=8$ Experiments were repeated 3 independent times. # $p < 0.05$ control vs. treatment groups, * $p < 0.05$ vs. erastin only. One-way ANOVA, Tukey post-hoc test.

Response of Pancreatic β - Cell Function to Curcumin, EGCG and Erastin

When MIN6 pancreatic β cells were treated with erastin, and became subject to ferroptosis, insulin synthesis would be affected. As is shown in figure 3.5, secretory insulin levels were decreased after treatment of 20 μ M erastin in MIN6 cells. It is predicted that polyphenol treatment might restore β cell functions in ferroptosis. 20 μ M curcumin treatment increased the insulin production in MIN6 cells ($p < 0.05$). However, EGCG treatment did not show any effect on insulin secretion in MIN6 cells.

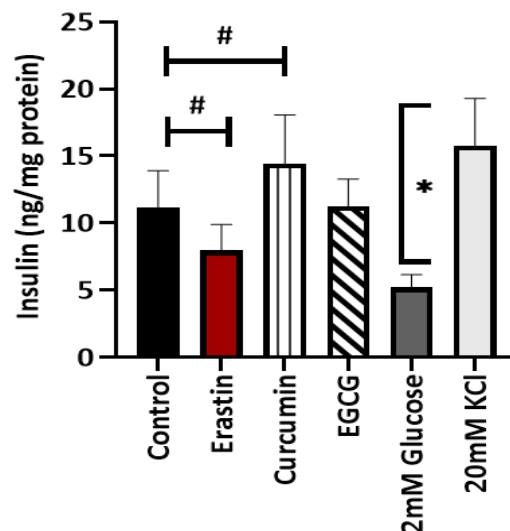


Figure 3.5. Curcumin improved the insulin secretion MIN6 cells. Cells were treated overnight with 20 μ M erastin, curcumin or EGCG. Insulin levels were measured in the cell supernatants. Insulin levels are relative to control cell samples. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ controls vs. treatment groups, * $p < 0.05$ 2 mM glucose concentration vs. 20 mM glucose concentration groups. One-way ANOVA, Tukey post-hoc test.

3.3. Discussion

Polyphenols offer beneficial pharmaceutical and medicinal properties (15,16) in addressing a range of ailments and disorders (16). However, much remains unknown regarding the mechanisms of action of most polyphenolic compounds. In addition, the specific effects of therapeutic flavonoids need to be investigated (16). Pancreatic cells are extremely susceptible to oxidative stress due to the production of high levels of endogenous ROS and a low expression of anti-oxidative enzymes (17).

Curcumin, quercetin, rutin, EGCG, tannic acid and phytic acid are widely used in the diet and show antioxidant and iron binding properties in several research (18). The current study investigated the protective effects of these different polyphenols on iron overload in a pancreatic β -cell model. Based on a variety of research, dose of polyphenols used in the antioxidant in vitro studies is at 20 μ M (19) which is the initial dose of polyphenols in this study. The effects of iron stressors (Fenton Substrate and 8HQ+FAC, iron loading) on MIN6 cell viability were tested in vitro. FS and 8HQ+FAC exerted pronounced toxicity in the cultured cells. However, both curcumin and EGCG showed protective effects against iron-induced toxicity (Figure 1A, B). This is in agreement with the protection conferred by curcumin in brain cells cultured from Alzheimer's patients, where curcumin inhibited cell death and decreased ROS production (20). Moreover, Mandel *et al.* (21) showed a notable decrease in lipid peroxidation and oxidative modifications of membranes in neural cortex and hippocampal cells after exposure to epicatechin. The mechanisms underlying the prevention of damage by ROS and iron with curcumin and EGCG are unclear, but could involve binding of iron, prevention of redox cycling by iron, and quenching of free radicals formed by iron (22,23). These properties could contribute to effective neutralisation of iron toxicity.

The mechanisms of action of EGCG and curcumin are complex and likely to be multifaceted. Curcumin and EGCG are likely to modulate expression and activity of proteins of iron metabolism, suggesting that these polyphenols have abilities of an iron chelator (23,24). Under conditions of iron deprivation, iron regulatory proteins (IRPs) are activated to repress ferritin and stabilise transferrin receptor 1 mRNA (TfR1). Activation of IRPs, translational repression of ferritin, and induction of TfR1 serve as indicators of reduced intracellular iron and are observed in cells treated with iron chelators. Both activated IRPs and TfR1 increased in response to curcumin in mice (25).

Furthermore, curcumin or EGCG co-treatment reversed cell death triggered by 20 μ M erastin that was evident at 24, 48, and 72 h (Figure 2). The polyphenols also suppressed iron accumulation that was mediated by erastin treatment in pancreatic cells (Figure 3.3A), corroborating previous findings which demonstrated that baicalein decreased iron levels in PANC1 and BxPc3 cells against erastin-induced ferroptosis (26). Moreover, Dixon *et al.* (27) showed that DFO and ferrostatin-1, potent inhibitors of ferroptosis, prevented iron accumulation and iron dependent-ROS production in breast cancer cells.

Lipid peroxidation is one of the critical signalling events that characterize ferroptosis (27). There is a strong relationship between iron and lipid peroxidation, as iron is known to cause ROS generation in ferroptosis (28). Accordingly, it has been suggested that iron chelators and polyphenols might play a protective role against lipid peroxidation (26,27). The study also demonstrated that both curcumin and EGCG exerted a decrease in MDA accumulation in pancreatic cells treated with erastin (Figure 3.3B). Xie *et al.* (26) showed that baicalein inhibited lipid peroxidation in pancreatic cancer cells treated with erastin. Furthermore, L6 skeletal muscle cells treated with EGCG, and docosahexaenoic acid (DHA) exhibited lower ROS production, and lower MDA levels, than cells treated with DHA alone (29). Together, these studies suggest that polyphenolic compounds by acting as ROS scavengers can limit lipid peroxidation of cellular membranes. Compelling evidence shows that when repair mechanisms such as antioxidant defence enzymes are overwhelmed, cell death in response to iron accumulation and lipid peroxidation becomes unavoidable.

Reduced GSH is an essential intracellular antioxidant with a vital role in the protection of lipids and DNA from ROS (27). In ferroptosis, erastin treatment leads to inhibition of system Xc⁻, which is the oxidative stress-inducible cysteine-glutamate exchange system, thus causing the inhibition of GSH biosynthesis (30,31). Curcumin is known to induce phase II antioxidants enzymes such as glutathione reductase, glutathione-S-transferase, glutathione peroxidase, NAD(P)H: quinone oxidoreductase-1, and thioredoxin reductase (24). It is shown in this study that erastin-induced GSH depletion was ameliorated by curcumin and EGCG in MIN6 cells (Figure 3.4A). This in turn could potentially influence the antioxidant activity of glutathione peroxidase 4 (GPX4), apart from the direct suppression of its protein level by erastin (Figure 3.4C) (31).

In agreement with the current study, breast cancer cells treated with erastin caused GSH depletion, which inactivated GPX enzymes and induced ferroptosis (32). Interestingly, α -tocopherol complemented GPX4 deficiency and prevented cell death in GPX4 knockout mice (33). In essence, GPX4 is one of the most important membrane protective enzymes. Erastin-induced GPX4 degradation in neurons and T cells accelerated lipid peroxidation and ferroptotic cell death. GSH levels decreased in the same study, but not to the level of statistical significance (34). Consequently, erastin could bind directly to GPX4 to reduce its enzymatic activity, or indirectly reduce the level of glutathione in the cells (27,28).

Curcumin is a natural polyphenol that has shown great potential as a novel therapeutic agent due to its pharmacological safety and efficacy in treating a wide variety of human diseases. As an antioxidant, curcumin has been shown to reverse streptozotocin-induced glucose tolerance, hypoinsulinemia, and damage of pancreatic islets (35). The level of insulin in cell culture models is used as a biochemical marker to predict pancreatic damage and to assess the impacts of natural polyphenols (36). This study showed that erastin-related ferroptosis is associated with decreased insulin release whereas curcumin treatment increased the insulin secretion in glucose-stimulated MIN6 cells. In comparisons, the inhibitory effects of insulin secretion in pancreatic β cells by polyphenols were reported (37). Excess doses of iron chelators and free radical scavengers were ineffective in relieving ROS and were found to be toxic to rat pancreatic cells. Based on the concentration of natural polyphenols, it might act as an either as a potential prooxidant or antioxidant. Additionally, the type of pancreatic β cell line involved in insulin secretion would need to be a factor.

Curcumin has a long history of consumption as part of the normal human diet. Extensive scientific research over the past decades has shown that curcumin has a variety of pharmacological activities such as anti-oxidative, anti-inflammatory, and anticancer without producing adverse health effects in dietary intake levels. However, despite its showed effects, the health benefits of curcumin are restricted by its low oral bioavailability, which can be attributed to the high rate of metabolism, poor absorption, and rapid systemic elimination from body (38). The results of a pharmacokinetic study conducted by Yang *et al.* (39) presented that a highest serum concentration of 0.36 $\mu\text{g/mL}$ was showed after intravenous administration, whereas a maximum plasma concentration of 0.06 $\mu\text{g/mL}$ was achieved for orally supplemented curcumin. The comparison showed that oral bioavailability of curcumin

was almost 1%. Several studies have examined the pharmacokinetic profile of curcumin in humans and animals. The highest plasma concentrations reported are 0.051 $\mu\text{g/mL}$ from 12 g curcumin intake in human, 0.22 $\mu\text{g/mL}$ from 1 g/kg in mouse and 1.35 $\mu\text{g/mL}$ from 2 g/kg in rat (40). Also, curcumin has achieved the status of Generally Recognized as Safe by the Food and Drug Administration (FDA), thus it has been safely administered at high doses in various clinical trials (41). Because curcumin has limitations such as absorption and lower water solubility, several approaches have been explored to improve its bioavailability. First, phospholipid complexation is a possible way for increasing the bioavailability of curcumin. The amphiphilic nature of this complex can improve water and lipid solubility, thus enhancing the absorption. In the study of Liu *et al.* (42), phospholipid-curcumin complex was prepared and orally administered to Sprague-Dawley male rats at dose of 100 mg/kg. Curcumin–phospholipid complex showed a plasma curcumin level of 0.6 $\mu\text{g/mL}$ while free curcumin showed plasma concentration of 0.267 $\mu\text{g/mL}$. In addition to that, lipid nanoparticles are particularly useful in the delivery of water-insoluble drugs because their small size (structures less than 100 nm in size) can increase the solubility and absorption and thus enhancing the bioavailability of the delivered drug. In the study of Gota *et al.*(43), healthy volunteers administrated at 650 mg/kg dose of solid lipid curcumin particles showed curcumin plasma levels of 0.025 ng/mL, whereas free curcumin (95% curcuminoids extract) at the same dose was undetectable. It is still not clear how curcumin nanoparticles increase the bioavailability due to an increased absorption or reduced conversion of free curcumin to conjugates.

The biological activity of EGCG including ROS scavenging and metal chelation capacity comes from a variety of active phenolic hydroxyl groups in its molecular structure. However, the bioavailability of oral EGCG intake from diet is relatively poor because of instability in the gastrointestinal tract and low intestinal absorption. It has been reported that the highest plasma concentration of EGCG was only 0.15 μM after two cups of green tea in human (44). EGCG has been extensively studied in nanoparticles to augment its bioavailability. Phospholipid based nanoparticles have been investigated, and absorption of EGCG in Caco-2 intestinal cells was increased compared with the EGCG treatment group (45). Therefore, novel delivery strategies present significant promise and are worthy of further exploration to enhance the oral bioavailability of EGCG and curcumin.

The results of this study revealed that (i) curcumin or EGCG treatment reversed the cell death triggered by erastin, (ii) curcumin and EGCG ameliorated erastin-induced iron accumulation, (iii) the levels of lipid peroxidation in MIN6 cells were decreased by treatment with curcumin or EGCG, and (iv) curcumin and EGCG attenuated erastin-induced decrease in GSH and GPX4 protein levels in the cells. The study shows that curcumin and EGCG are inhibitors of ferroptosis in cultured MIN6 pancreatic β -cells and may protect against iron-induced oxidative damage in iron-overload disorders.

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Chapter 4. Protective effect of Ferulic acid against ferroptosis-induced murine MIN6 cell damage by activation Nrf2 pathway

Abstract

Ferroptosis is a regulated cell death process characterised by iron-dependent accumulation of oxidized polyunsaturated fatty acid containing phospholipids. Its initiation is complicated and involves in reactive oxygen species (ROS) and loss of activity of the lipid repair enzyme glutathione peroxidase 4 (GPX4). These play critical roles in the development of ferroptotic cell damage by lipid peroxidation. Antioxidant therapy is a promising therapeutic strategy to prevent or even reverse the progression of ferroptosis. This study was designed to demonstrate the protective effect of ferulic acid (FA) against oxidative stress and erastin-mediated ferroptosis in murine MIN6 cells. Cells were treated with FA and its metabolite ferulic acid 4-O-sulfate disodium salt (FAS) and 20 μ M erastin. Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay, iron levels were measured by inductively-coupled plasma mass spectrometry (ICP-MS), ROS levels were determined by dihydrodichlorofluorescein (H2DCF) cell-permeant probe, glutathione and lipid peroxidation were assayed with commercially-available kits. The phenolic acids enhanced cell viability in erastin-treated MIN6 cells in a dose dependent manner. Furthermore, MIN6 cells exposed to erastin alone showed elevated levels of iron and ROS, glutathione (GSH) depletion and lipid peroxidation ($p < 0.05$) compared to cells that were protected by co-treatment with FA or FAS. Treatment of MIN6 cells with FA and FAS following exposure to erastin increased the nuclear translocation of Nrf2 protein levels. Consequently, levels of its downstream antioxidant proteins; HO-1, NQO1, GCLC and GPX4 increased. FA and FAS greatly decreased erastin-induced ferroptosis in the presence of Nrf2 inhibitor, ML385 through regulation of Nrf2 response genes. In conclusion, these results show that FA and FAS protect MIN6 cells from erastin-induced ferroptosis by Nrf2 antioxidant protective mechanism.

4.1. Introduction

Iron is vital for several biologic processes whereas excess-iron results in oxidative stress, subsequent mitochondrial impairment, DNA damage, lipid peroxidation, leading in particular

to dysfunction of metabolically highly active cells such as hepatocytes, cardiomyocytes and pancreatic β cells (1).

Growing body of evidence suggests that iron-induced pancreatic β cell impairment is involved in T2D and/or its complications (2,3) including insulin synthesis and β cell signalling pathways (4). Insulin-producing β cells are known to have deficient antioxidant defence status and are highly susceptible to free radical-induced oxidative damage (5). Besides, pancreatic β cells have highly active mitochondria, which incidentally generate activated ROS, capable of leading to synergistic toxicity with intracellular iron, especially accentuated by intrinsically weak antioxidant defences (6).

Ferroptosis, as a form of regulated cell death, that is characterised by the accumulation of iron-based lipid reactive oxygen species (ROS). Erastin as a ferroptosis inducer which targets to block glutamate-cystine antiport system Xc⁻ and thus inhibiting the exchange of extracellular cysteine and intracellular glutamate which are necessary components of glutathione protein (7). Glutathione is an important antioxidant capable of removing ROS and protecting cells from oxidative stress. Lack of glutathione can activate lipoxygenase, inhibit GPX4 activity and induce lipid peroxidation, thus leading to the disruption of cell structure, that results in cell death (8).

Ferroptosis plays a crucial role in pancreatic β cell death due to excess iron exposure and its low antioxidant reserve (9). Importantly, cells have a numerous endogenous antioxidant defence mechanism in place to keep the level of reactive species in check, including the basic leucine zipper (bZIP) transcription factor, nuclear factor E2 related factor 2 (Nrf2) which attenuates ROS and oxidative stress in cells. This factor is a vital regulator of the cellular antioxidant response, controlling the expression of genes such as, HO-1, NQO1, GCLC and GPX4 that counteract oxidative and electrophilic stresses (10). As such, proper Nrf2 functionality is critical for cell survival under increased metabolic and oxidative stress. Due to its multifaceted role in promoting cell survival, it is not surprising that inhibition of Nrf2 and/or its downstream target genes are associated with a decreased responsiveness to cellular stressors and increased cell death, ferroptosis (11).

In this research, FA was used due to low toxicity and high bioavailability rate compared to other phenolic acids. Furthermore, FA is a plant-based phenolic acid that is commonly found in fruits, vegetables and some beverages including coffee and beer (12). FA has been reported to exhibit antioxidant, anti-inflammatory, anticancer and hepatoprotective effects (13). Its antioxidant property is due to the phenolic hydroxyl group in its structure that donates electrons to quench free radicals (14). Furthermore, FA functions as an Nrf2 agonist and can promote the nuclear translocation and biological effects of Nrf2 (15).

This study aimed to investigate the functions of FA and its metabolite FAS, which protected MIN6 cells against ferroptosis via promoting cell viability and decreased iron and lipid peroxidation levels. The molecular antioxidant mechanism was associated with the activation of Nrf2 pathway. These results suggested that FA and FAS may be considered as a therapeutic agent for the treatment and prevention of ferroptosis in MIN6 pancreatic β cells.

4.2. Results

FA and FAS alleviate decreased viability of MIN6 cells induced by erastin.

Protective effects of FA and FAS on MIN6 cells were investigated by MTT assay, the results indicated that FA and FAS (5-100 μ M) did not cause any obvious cytotoxicity (Figure 4.1A). Next, MIN6 cells were exposed to a series of erastin concentrations for 24 h. It is found out that erastin exposure reduced cell viability in dose dependent manner (Figure 4.1B). An exposure of MIN6 cells to 20 μ M erastin for 24 h resulted in a significant ($p < 0.05$) 38 % cell death than the untreated control group. Therefore, 24 h treatment with 20 μ M erastin was used to induce ferroptotic injury to MIN6 cells in this study.

To examine the protective effect of FA and FAS treatments on erastin-induced ferroptosis in MIN6 cells, cells were treated with or without FA and FAS (5, 10, 20, 30 and 40 μ M) and 20 μ M erastin for 24 h. Concentrations of phenolic acids were determined based on the research related with antioxidant effects of FA. Erastin reduced cell viability significantly ($p < 0.05$) which was abolished by 20 μ M FA and FAS treatment. These findings indicate that 20 μ M FA and FAS can reduce erastin-induced cell toxicity (Figure 4.2B and 2C). Moreover, MIN6 cells were treated with different concentrations of ferroptosis inhibitor, Fer-1 in the presence of 20 μ M erastin. Fer-1 a ferroptosis inhibitor, even at 1 μ M concentration reverted cell death caused

by 20 μM erastin (Figure 4.2A). It is demonstrated that ferroptosis inhibitor, Fer-1 co-treatment could significantly restored MIN6 cell viability when subjected to death triggered by 20 μM erastin-induced ferroptosis.

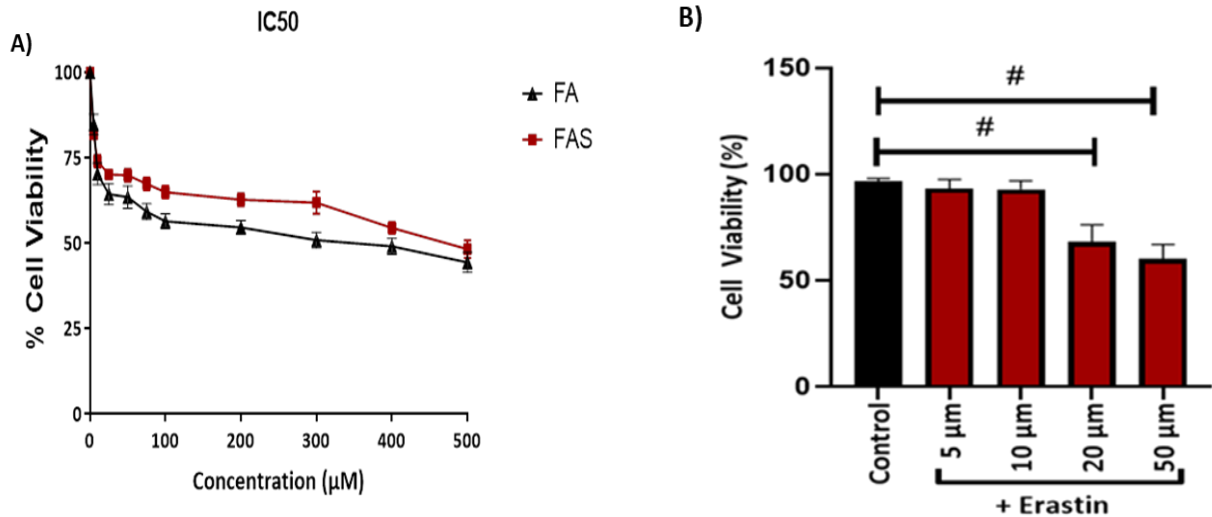


Figure 4.1. Relative cell viability of MIN6 cells against different concentrations of FA, FAS or erastin.

A) MIN6 cells were exposed the different concentrations of ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) for 24 h to determine IC50 concentration. Cell viability was measured by MTT assay. Data are presented as the percentage of cell viability with the error bars represent the SEM. B) MIN6 cells were treated with the indicated concentrations (5, 10, 20 and 50 μM) of ferroptosis inducer, erastin for 24 h to show cell death by MTT assay. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control vs. treatment groups, One-way ANOVA, Tukey post-hoc test.

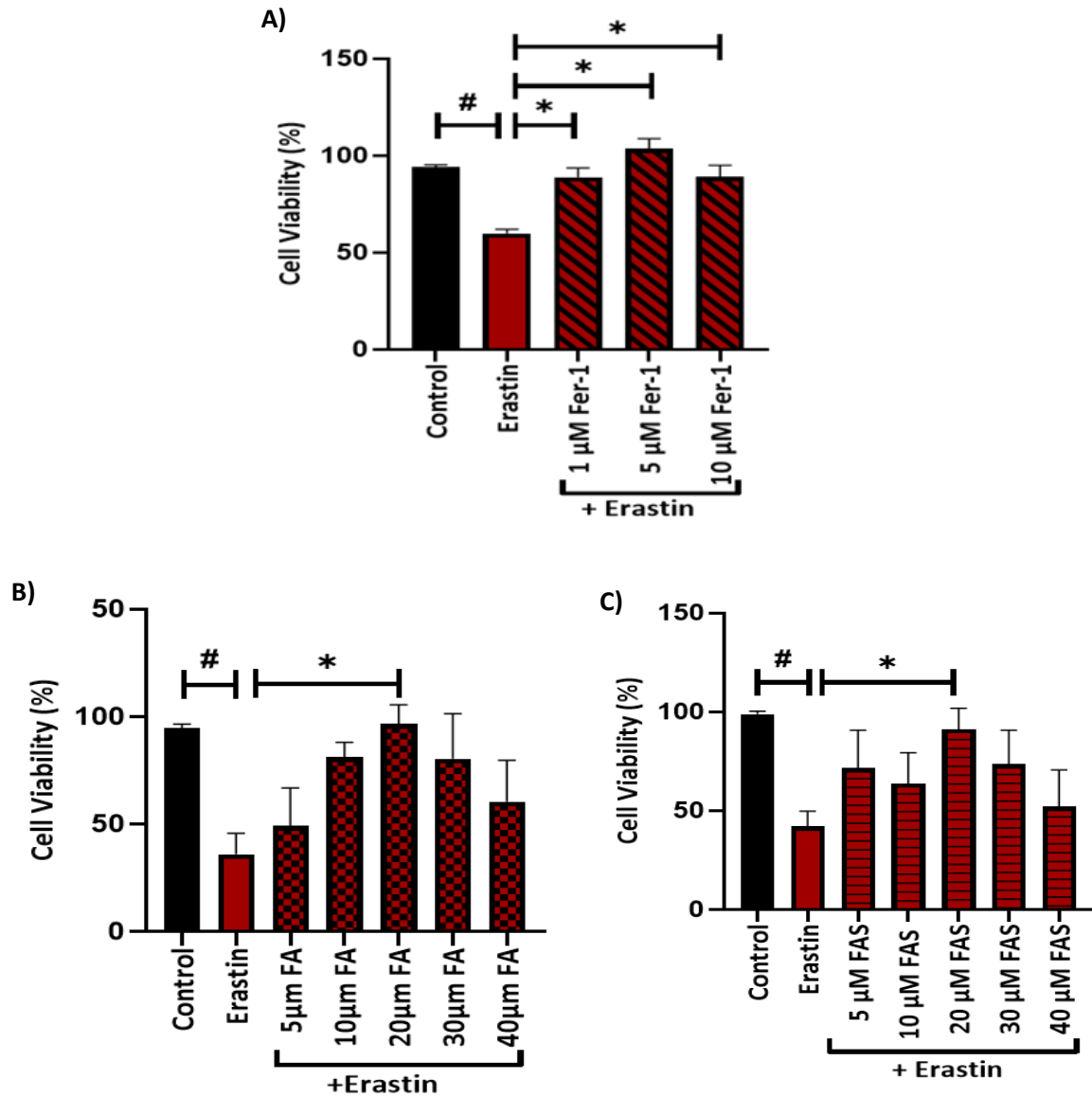


Figure 4.2. FA and FAS protect MIN6 cell viability from erastin-induced ferroptosis. MIN6 cells were exposed to ferroptosis inducer, 20 μ M erastin in the absence or presence of (5, 10, 20, 30 and 40 μ M) ferulic acid (FA) or, (5, 10, 20, 30 and 40 μ M) ferulic acid 4-O-sulfate disodium salt (FAS) or (1, 5 and 10 μ M) Ferrostatin (Fer-1) at the dose dependent manner for 24 h. Cell viability was assayed to show protective effects of FA, FAS or Fer-1 against erastin treatment. All the values are expressed with the mean $\pm$ SEM, n = 8. Experiments were repeated 3 independent times. #p < 0.05 control vs. treatment groups, *p < 0.05 erastin vs. treatment groups One-way ANOVA, Tukey post-hoc test.

FA and FAS suppress iron accumulation and can act as iron chelators.

Next, the role of iron in ferroptosis was studied. ICP-MS analysis showed that the concentration of iron in MIN6 cells increased after 20 μ M erastin treatment by 0.3 nm/mg which was inhibited by co-treatment of 20 μ M FA and FAS and 20 μ M erastin (Figure 4.3A). To validate the effect of FA and FAS on cellular iron levels, it is important to elucidate their mechanism as iron chelators. Since cell death caused by ferroptosis is highly related to free iron *in vivo*, hence iron chelation is inhibitory to ferroptosis which is inhibited (19). Probably, polyphenols might also function similarly. As shown in Figure3B, FA and FAS resulted in 46% iron chelation ability in low concentration and 50% in high concentration while that of FA was 42% in low concentration and 49% in high concentration. This showed that both FA and FAS have similar iron chelation abilities. Also, these polyphenols indicated an upward trend of chelating potential with the increase dose (Figure 4.3B).

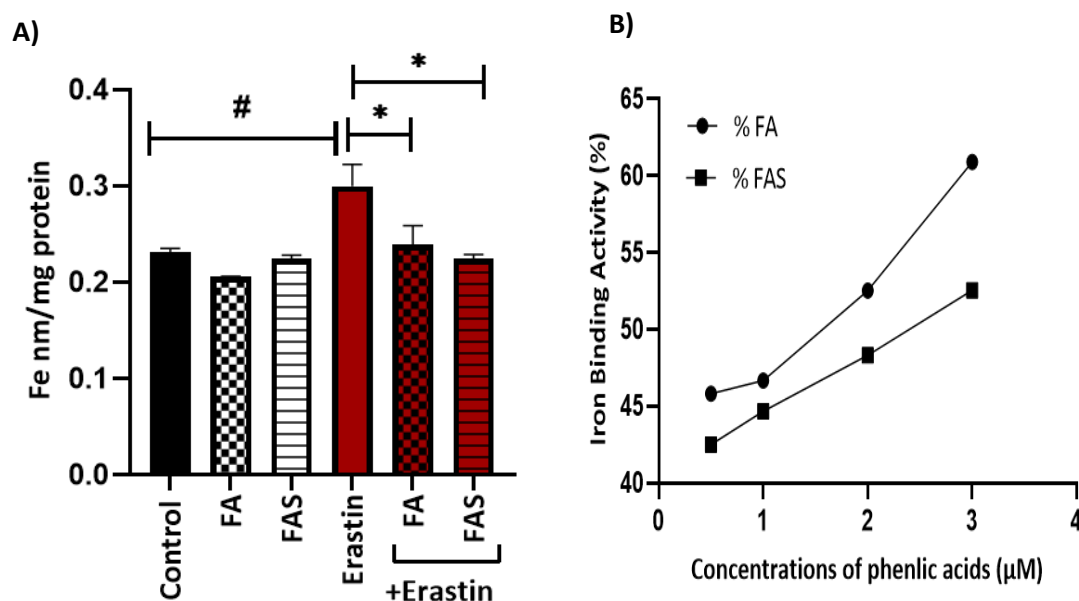


Figure 4.3. Treatments of FA and FAS reduce iron concentrations by having abilities of iron chelator.

A) Cells were cotreated with 20 μ M erastin in the absence and presence of 20 μ M ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) for 24 h. Then, iron concentrations were measured by ICP-MS analysis. B) Different concentrations of FA and FAS (0.5 μ M, 1 μ M, 2 μ M and 3 μ M) were analysed to show iron chelation ability. All the values are expressed with the mean $\pm$ SEM, n = 8. Experiments were repeated 3 independent times. #p<0.05 control vs. treatment groups, *p < 0.05 erastin only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Treatments of FA and FAS limit lipid peroxidation and ROS production by increasing GSH antioxidant levels.

To examine whether FA and FAS can protect MIN6 cells under ferroptosis damage, we built the ferroptotic damage model of pancreatic MIN6 cells, which was made by treating cells with 20 μ M erastin. Intracellular ROS level was detected by using ROS probes using flow cytometry in erastin-treated MIN6 cells. Compared with the control group, the intracellular ROS level of MIN6 cells was significantly increased by 1.01 folds after the treatment with erastin. Notably, cotreatment with FA and FAS effectively reduced erastin-induced ROS burden by nearly 0.79, 0.71 folds, respectively, although a single treatment with FA and FAS did not change intracellular ROS level in MIN6 cells (Figure 4.4A). MDA is an index of membrane lipid peroxidation, which indirectly reflects the degree of damage to a cell (20). In order to validate the effects of FA and FAS on the antioxidant capacity of erastin-induced MIN6 cell injury model, GSH and MDA levels were measured. As shown in Figure 4.4B, when compared with the erastin alone treatment group, MDA levels in FA or FAS and erastin co-treatment groups were significantly decreased by 4.7 and 5.0 nmol/mg protein respectively. GSH plays a crucial role in restoring cell function through removing lipid peroxides, H_2O_2 , $O_2^{\cdot-}$ and LOOH. During ferroptosis, Fe^{2+} converts membrane lipids into hydroperoxides, and GPX4 converts lipid peroxides into the corresponding alcohol using GSH as a cofactor. In figure 4.4C, erastin alone treatment group decreased GSH levels by 42 nmoles/ml whereas FA and FAS treatment in MIN6 cells exposed to erastin increased GSH levels by 61 and 66 nmoles/ml, respectively. Therefore, it could be concluded that FA and FAS downregulate the level of ROS by promoting GSH and inhibiting Fe^{2+} and MDA levels, thereby alleviating ferroptosis.

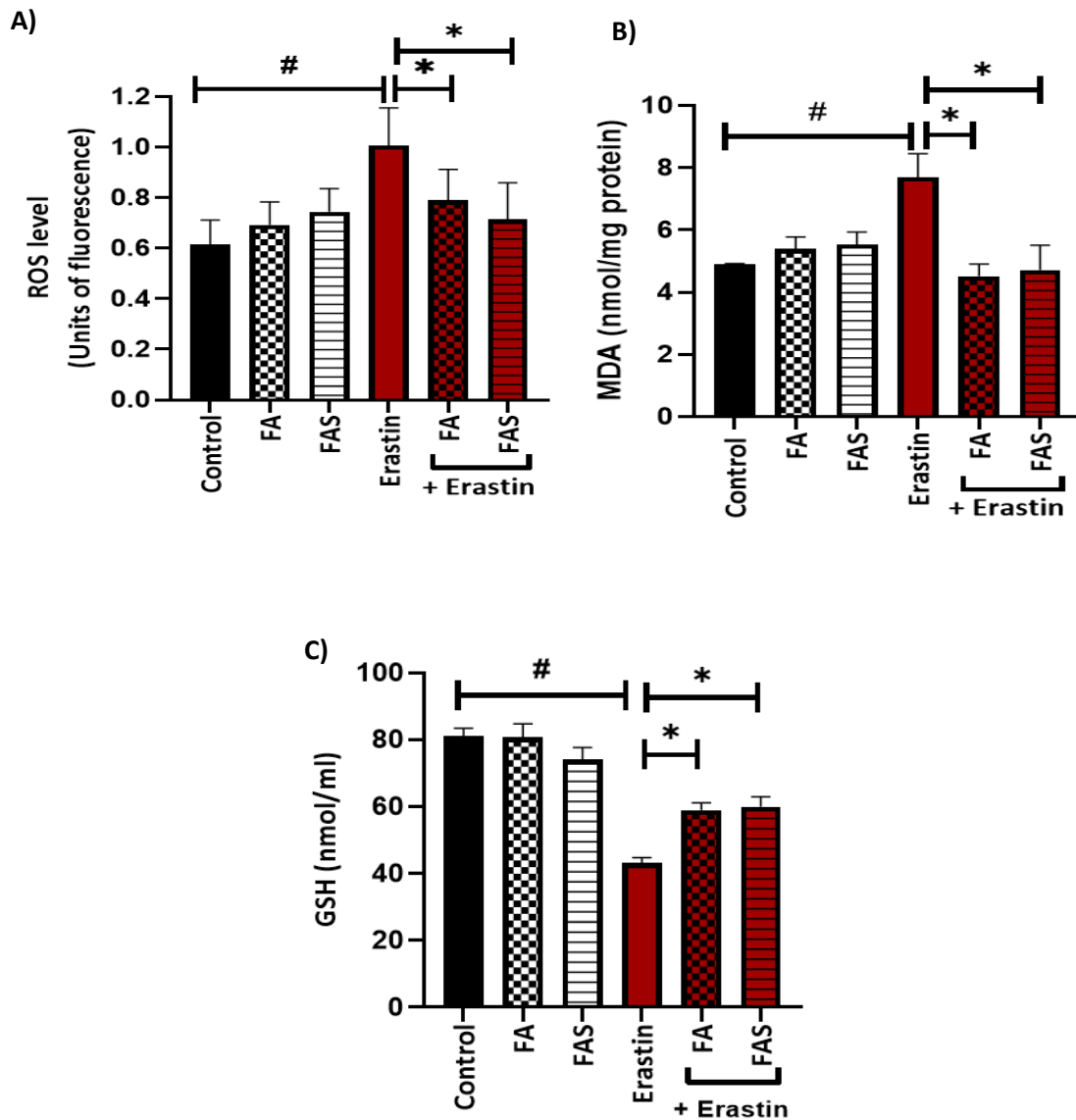
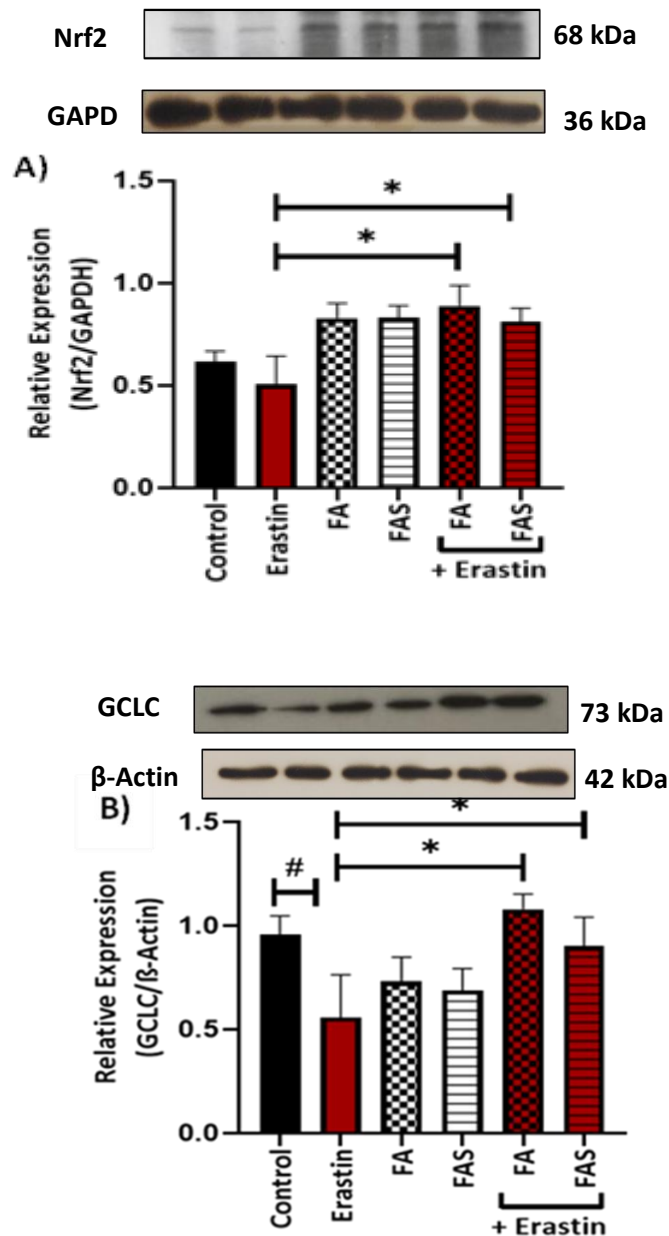


Figure 4.4. FA and FAS limit lipid peroxidation and ROS production by increasing GSH levels. Cells were cotreated with 20 μ M erastin in the absence and presence of 20 μ M ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) for 24 h. Then, cellular reactive oxygen species (ROS) by dihydrodichlorofluorescein (H2DCF) cell-permeant probe (A), lipid peroxidation levels by Malondialdehyde (MDA) assay (B) and glutathione (GSH) amounts by total GSH assay (C) were measured. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control vs. treatment groups, * $p < 0.05$ erastin only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Activation of Nrf2 signalling supresses erastin-induced ferroptosis by FA and FAS treatment.

To further analyse the mechanism of the cytoprotective role of FA and FAS on erastin-induced MIN6 cell injury, protein levels of nuclear form of Nrf2, and GPX4, HO-1, GCLC, NQO1 were determined. Erastin alone treatment significantly reduced the protein expression levels of Nrf2, GPX4, HO-1, GCLC and NQO1 ($p < 0.05$) (Figure 4.5). On the contrary, treatment with FA and FAS significantly increased the expression of nuclear Nrf2, GPX4, HO-1, GCLC and NQO1 in erastin-induced cells. These results suggested that FA and FAS can activate Nrf2 signalling pathway.



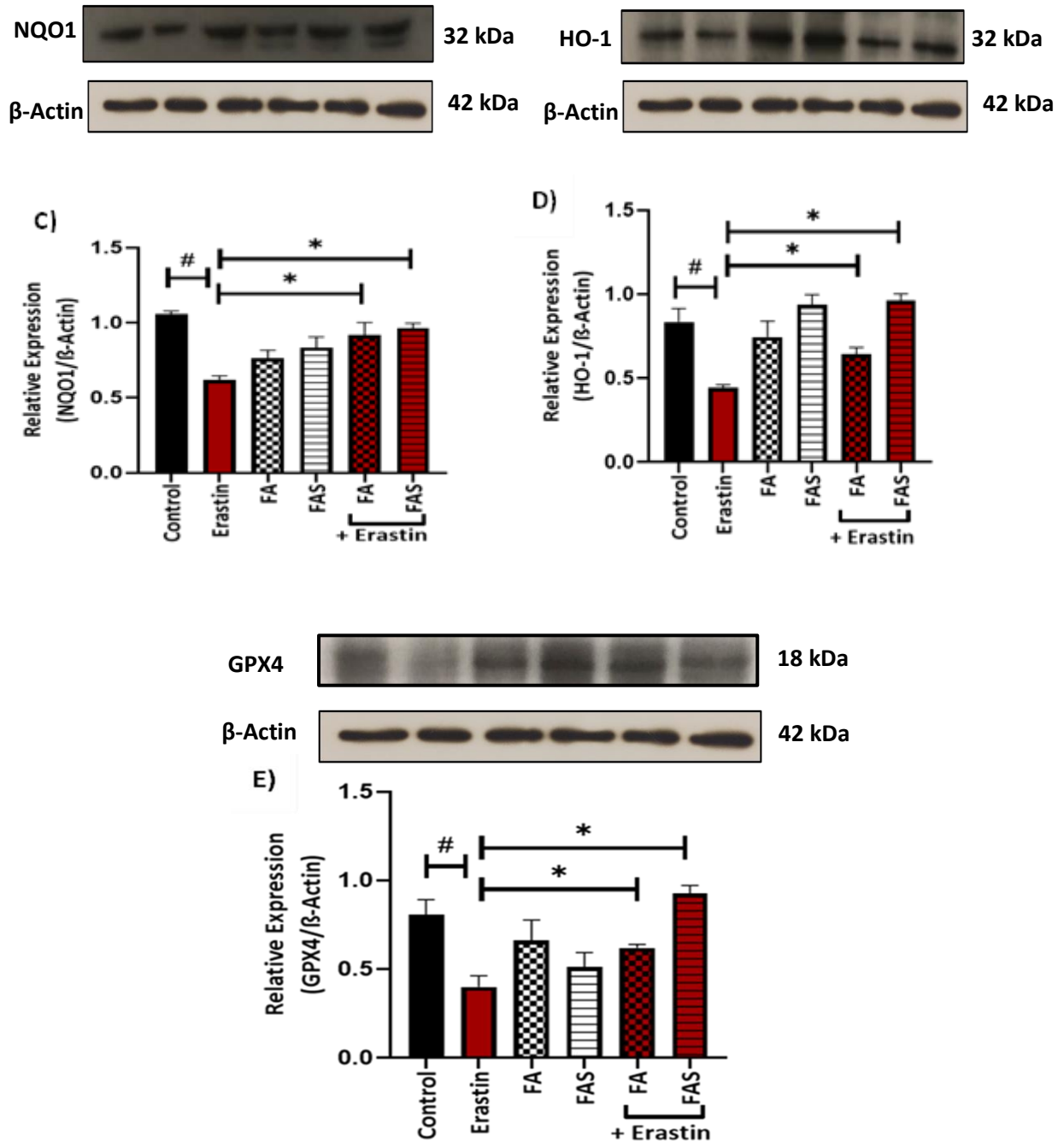


Figure 4.5. FA and FAS can activate Nrf2 pathway. Cells were cotreated with 20 μ M erastin in the absence and presence of 20 μ M ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) for 24 h. Total cell lysates were prepared and subjected to Western blot analysis to monitor the expression levels of nuclear Nrf2 and its downstream antioxidant proteins including NQO1, GCLC, GPX4 and HO-1. GAPDH and β -Actin served as an internal control. The representative figure shows one of the three independent experiments. Quantitative results of the protein expressions in pancreatic β cells determined by greyscale analysis of using the ImageJ software. All the values are expressed with the mean $\pm$ SEM, # p < 0.05 control vs. treatment groups, * p < 0.05 erastin only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Nrf2 activation shows the protection against ferroptosis in ML385 treated MIN6 cells.

To further indicate the involvement of Nrf2 on the protective effect of FA and FAS in MIN6 cells under ferroptosis, it was established MIN6 cells treated with 10 μ M ML385 for 2h, a Nrf2 inhibitor. The results revealed that ML385 when combined with erastin treatment significantly reduced cell viability by 38% compared with control group ($p<0.05$) (Figure 4.6A). Furthermore, FA and FAS alleviated the toxic effect of erastin in ML385 pretreated MIN6 cells. In terms of antioxidant role of FA and FAS, GSH and MDA levels were detected. FA and FAS showed the repressive effects on lipid peroxidation levels in erastin-induced MIN6 cells in the presence of ML385 pre-treatment, which decreased by 8 and 7 nmol/mg protein, respectively ($p<0.05$) (Figure 4.6B). In addition, erastin treatment after pre-treatment of MIN6 cells with ML385 for 1 h decreased GSH levels by 25 nmoles/ml compared with control group while FA and FAS co-treatment with erastin augmented GSH levels by 38 and 40 nmoles/ml, respectively in ML385 pre-treatment ($p<0.05$) (Figure 4.6C). After pre-treatment of MIN6 cells with ML385, protein levels of Nrf2, and its downstream genes including GCLC and NQO1 were increased in erastin, FA or FAS co-treatment groups compared with erastin alone ($p<0.05$) (Figure 4.7). Collectively, these data indicate that the cytoprotective effect of FA and FAS against ferroptosis can be achieved by activating Nrf2.

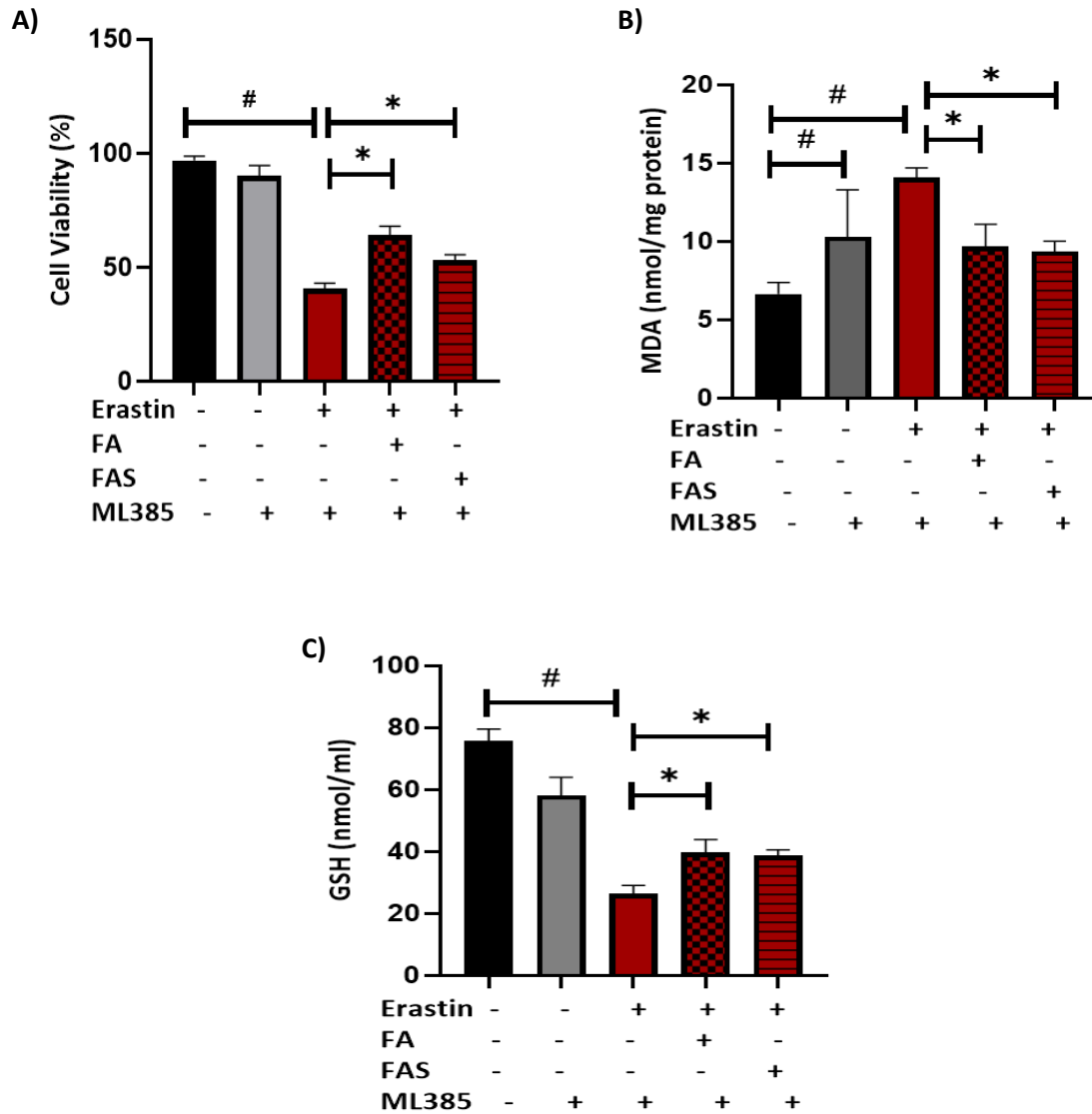


Figure 4.6. Phenolic acids protect MIN6 cells treated with ML385, Nrf2 inhibitor against ferroptotic markers. Cells were treated with 10 μ M Nrf2 inhibitor, ML385 for 2h before ferroptosis treatment. Then, MIN6 cells were exposed to 20 μ M erastin in the absence and presence of 20 μ M ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) for 24 h. Cell viability was presented by MTT assay (A), lipid peroxidation was analysed by MDA assay (B) and total glutathione (GSH) amount (C) was measured by GSH assay. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. $^{\#}p < 0.05$ control vs. treatment groups, $^*p < 0.05$ erastin only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

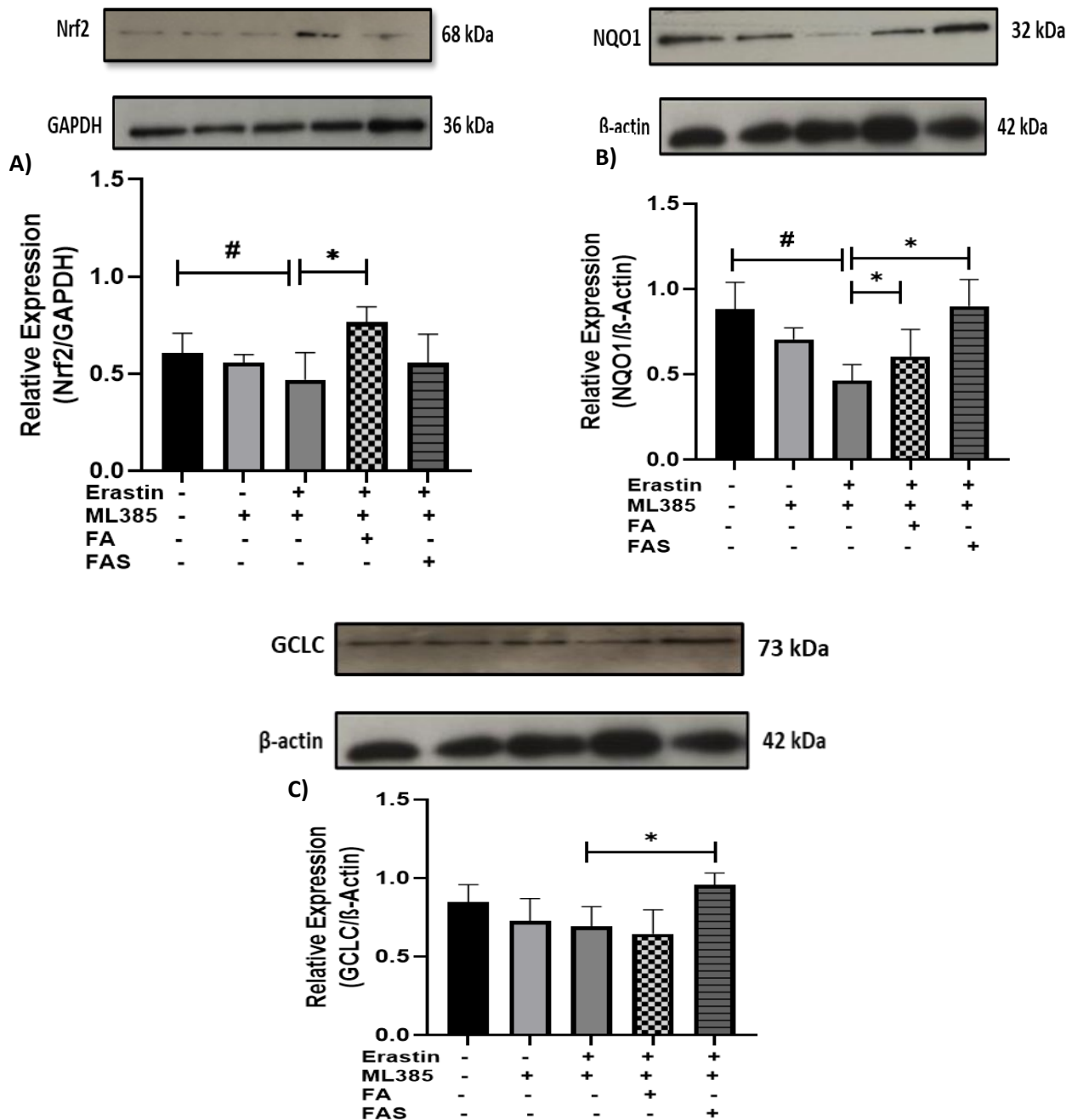


Figure 4.7. Nrf2 activation shows the protection against ferroptosis in ML385 treated MIN6 cells. Cells were treated with 10 μ M Nrf2 inhibitor, ML385 for 2h before ferroptosis treatment. Then, MIN6 cells were exposed to 20 μ M erastin in the absence and presence of 20 μ M ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) for 24 h. The expressions of Nrf2, GCLC and NQO1 were measured by Western blotting. GAPDH and β -Actin served as an internal control. The representative figure shows one of the three independent experiments. Quantitative results of the protein expressions in pancreatic β cells determined by greyscale analysis of using the ImageJ software. All the values are expressed with the mean $\pm$ SEM, # p < 0.05 control vs. treatment groups, * p < 0.05 erastin only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Responses of phenolic acids and ferroptosis inducer on insulin secretion in MIN6 cells.

As shown in Figure 4.8, 20 μ M FA induced a significant increase (about 1.5-fold) in insulin secretion. Under similar experimental conditions, the depolarizing agent KCl (20 mmol/l) as a positive control provoked a 4.2-fold increase compared with low glucose level group ($p < 0.05$). Ferroptosis inducer, erastin decreased insulin secretion ($p < 0.05$).

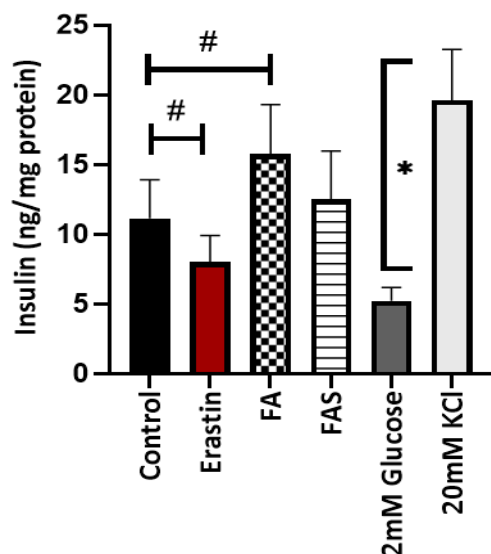


Figure 4.8. Phenolic acids and ferroptosis inducer affect insulin secretion in MIN6 β cells. Mouse MIN6 β cells were treated with 20 μ M ferulic acid (FA) or ferulic acid 4-O-sulfate disodium salt (FAS) or ferroptosis inducer, erastin for 3 h under 20 mmol/l glucose conditions. Supernatants from triplicate samples were analyzed for responses of insulin secretion in MIN6 cells. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ controls vs. treatment groups, * $p < 0.05$ 2 mM glucose concentration vs. 20 mM glucose concentration groups. One-way ANOVA, Tukey post-hoc test.

4.3. Discussion

Novel therapeutic measures are employing natural products that might offer protection against side effects of synthetic medications in the treatment of cancer, metabolic disorders and T2D. Polyphenols provide a new perspective for developing novel approaches against ferroptosis-induced cell damage. Induction of the antioxidant pathway, such as Nrf2 pathway, polyphenols are considered as a promising therapy to prevent ferroptosis. Therefore, this study investigated the underlying mechanisms about how FA has cytoprotective effects against ferroptosis via stimulating Nrf2 pathway.

Pancreatic MIN6 cells treated by erastin is a simple and feasible in vitro cell model to study ferroptosis, which can be effectively recovered by natural antioxidants. Previous reports have exerted that FA is an effective scavenger of free radicals such as, (OH), peroxy radical (RO₂) and hypochlorous acid (HOCl) and it has also been approved in certain countries as a food additive to prevent lipid peroxidation (21). Importantly, FA prevented erastin-induced ferroptotic cell death in MIN6 cells, in fact almost at the same level of Fer-1 (Figure 4.2), suggesting that FA can exert a strong antioxidant activity to neutralize the effect of ferroptosis inducers.

Ferroptosis results from iron-dependent lipid peroxide accumulation; thus, iron chelation therapy is a protective approach to prevent ferroptotic activity in cells. Indeed, free iron is the key factor that takes part in both iron metabolism and the promotion of lipid peroxide accumulation, which could not only build a connection between ROS, lipid peroxide and iron metabolism but also provide a way of depressing the creation of ROS using iron chelators (22). This study showed that both FA and FAS had an increasing iron-chelating ability which was a dose-dependent. That was in accordance with the study composed by Ferlazzo *et al.* (20) In their study, two juice extracts from bergamot and orange, which were abundant in polyphenols, showed iron-chelating properties in epithelial cells A549 treated with 200 and/or 400 μM Fe³⁺ (23). Dixon *et al.* (24) showed that the iron chelator, 100 μM deferoxamine (DFO) co-treatment in fibrosarcoma HT-1080 cells abrogated erastin-induced lethality. Also, co-treatments of phenolic acids suppressed iron accumulation that was mediated by erastin treatment in MIN6 pancreatic cells (Figure 4.3A), corroborating previous

findings which demonstrated that baicalein decreased iron levels in PANC1 and BxPc3 cells against erastin-induced ferroptosis (25).

This study found that FA inhibited MDA levels caused by iron accumulation by promoting GPX4 expression (Figure 4.4). Inhibition of GPX4 initiates uncontrolled polyunsaturated fatty acid oxidation and fatty acid radical generation thereby causing ferroptotic cell death, suggesting that ferroptosis is triggered mainly by reduced detoxification of lipid peroxides by the enzymatic activity of GPX4 (26) or loss of this capacity (27). This study is consistent with our previous results that curcumin and EGCG exerted a decrease in MDA accumulation in pancreatic cells treated with erastin (28).

Increased level of GSH is an indication of Nrf2 activation; thus, GSH amount in cells may be considered a significant factor underlying the protection associated with Nrf2 activation. GSH not only produces reducing equivalents, which are necessary for the conversion of H_2O_2 and lipid peroxides to water lipid alcohols but also has an essential role in the protection of protein sulfhydrylation against oxidation (29). This study exerted that erastin blocked glutamate-induced downregulation of GPX4 by a decrease in GSH levels while FA and FAS treatments increased GSH levels and in turn GPX activity. Similar to this result, that level of GSH dropped dramatically with the addition of oxidative stress inducer tert-butyl hydroperoxide (t-BOOH) and the decrease of GSH was suppressed significantly after exposure to epicatechin (30).

Nrf2 regulation serves a key role in modulating antioxidant enzymes and is also an essential part of maintaining oxidative and antioxidative homeostasis and alleviating oxidative stress damage (28). NQO1, GCLC, GPX4 and HO-1 are some downstream genes of Nrf2 regulation, this has a significant role in protecting cells from oxidative stress (31). The present study proposed that the potential antioxidative mechanism of FA may comprise Nrf2 signalling pathway. This study investigated whether FA could activate Nrf2 in pancreatic MIN6 cells. The results suggest that the translocation of Nrf2 to the nucleus after FA treatment reversed the inhibitory effects of erastin on the expression of NQO1, GCLC, GPX4 and HO-1 in MIN6 cells. In addition, the effects of FA and FAS on MIN6 cells treated with ML385 (an inhibitor of Nrf2 activity) were investigated in the present study. The results revealed that ML385 exhibited no toxicity to MIN6 cells. ML385 can abolish the protective effects of FA and FAS. At the

molecular level, Nrf2 inhibitor ML385 enhanced the impact of erastin on inducing cell damage via increasing MDA level. Furthermore, FA and FAS showed an increased expression of Nrf2, NQO1, GCLC, GPX4 and HO-1 in MIN6 cells treated with ML385 against erastin-induced ferroptosis. Therefore, the activation of Nrf2 signalling may be closely associated with the protective effects of FA.

The level of insulin release in cell culture models can be used as a biochemical marker to predict iron related pancreatic damage and to assess the effects of natural or synthetic iron chelators (29). This study indicated that ferroptosis is associated with a decreased insulin secretion whereas FA could increase the insulin secretion in glucose-stimulated pancreatic MIN6 cells. A study by Saji *et al.* (30) showed that rice bran phenolic extracts significantly increased glucose-stimulated insulin secretion in INS-1E cells. However, increased concentration of rice bran phenolic extracts decreased insulin secretion, suggesting that determination of certain concentrations of natural products is vital as an antioxidant activity. In comparison, the inhibitory effect of insulin secretion in pancreatic β cell line by natural products was reported (31). High doses of iron chelators and free radical scavengers were found to be toxic to rat pancreatic cells. Accordingly, depending on the concentration of natural products, they can act either as a prooxidant or potential antioxidant. Additionally, this effect might be influenced by the redox state of iron. The type of glucose sensitive pancreatic insulinoma β cells would need to be stated.

The physiological importance of FA, and notably its antioxidant property, depends upon its availability for absorption and subsequent interaction with target tissues. The bioavailability of FA has been addressed in several studies that quantified urinary excretion with variable results: from low to high bioavailability (0.4–98%), in part depending on the food source (32,33). For instance, the consumption of cereal products, particularly the bran, FA presented a low bioavailability: 3% in humans (34), 2.5–5% in rat (35) and even lower, 0.4–0.5%, from corn bran in rat (36). The bioavailability of FA was somewhat higher from other food matrices such as tomato, 11–25% (37) or rye bread, 28% (38), while from beer, FA was highly bioavailable, 19–98% (32). The concentration of FA in plasma under normal physiological conditions is in the range 0.2–2 μ M FA (39). In order to further elucidate the potential role of

FA on health benefits, studies on the bioavailability of FA from its major natural matrices are needed.

In summary, the present study proposed the novel functions of FA, which protected MIN6 cells against ferroptotic damage induced by erastin through promoting cell viability. The molecular mechanism is associated with the activation of Nrf2 related the expression of antioxidant genes. These results suggest that FA might be considered as a therapeutic agent for the treatment and prevention of pancreatic cell death.

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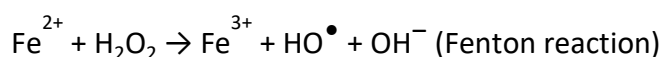
Chapter 5. Protective function of Ferulic acid against iron-induced stress in cell and mouse models

Abstract

Iron-related oxidative stress is a common denominator in the pathogenesis of insulin sensitivity or Type 2 diabetes (T2D). It is well known that the liver, a major site of iron storage, is particularly susceptible to injury from excess iron which leads to insulin sensitivity. Also, cell loss in pancreas resulted from high iron amount leads to an impairment of insulin secretion. Thus, antioxidant therapies are often used to reverse oxidative damage and to protect cells and tissues. The present study aimed to investigate the protective effects of phenolic acids; ferulic acid (FA) and its metabolite ferulic acid 4-O-sulfate disodium salt (FAS) against oxidative stress in iron overload conditions in mice and HepG2 cells. In the present study, phenolic acids exhibited remarkable protective effects to protect HepG2 cells which limited ROS production, lipid peroxidation and depletion of antioxidants and tissues against iron-induced oxidative stress. In vivo study showed the protective effects of both phenolic acids in major oxidative stress related tissues; reduction of iron accumulation, amelioration of antioxidant levels. Furthermore, the antioxidative effect of phenolic acids was achieved by the activation nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream antioxidative genes in liver and pancreas tissues. In conclusion, the study proves that FA could prevent insulin-related diseases from iron-induced oxidative stress by activating Nrf2. Therefore, phenolic acids have potentials as therapeutic agents for iron-related disease.

5.1. Introduction

Iron is involved in several cellular functions and processes in humans due to its role as an essential micronutrient. However, excess body iron levels are associated with increased level of oxidative stress that might cause cells and tissue injuries (1). Oxidative stress refers to the excessive production of reactive oxygen species (ROS) that iron can generate the highly reactive hydroxyl radical ($\text{OH}^\bullet$) through Fenton reaction associated with the damage on proteins, lipids, and nucleic acids. ROS exposure results in a loss of balance between the antioxidant system and the oxidation system, thereby leading to damage to healthy tissues, cells, and then causing organ dysfunctions and diseases, such as cancer, malignancies, neurodegenerative disease, diabetes and so on (2).



In cases of elevated iron levels, there is the largest iron store in several organs such as, liver, spleen, and kidney (2,3). Increased serum ferritin and body iron stores have been recognized as features of metabolic syndrome and T2D (4). The liver is particularly vulnerable to toxicants because of its high metabolic activity and its anatomical positioning to receive blood from the gastrointestinal tract. The absorbed drugs and xenobiotics in a concentrated form can lead to ROS and free radical-mediated damage that may cause inflammatory and fibrotic processes (5). Hepatic iron deposition has been detected in up to one-third of patients with nonalcoholic fatty liver disease (NAFLD), characterized by hepatic insulin resistance, which affects almost 20% of the general population, and is considered a manifestation of the metabolic syndrome (6). A reciprocal interaction of iron metabolism and insulin has been suggested to explain these findings. Genetic and acquired factors lead to increased iron absorption and storage in hepatocytes, with consequent alteration in the redox status. Excess iron reduces the hepatic extraction and the metabolism of insulin, leading to peripheral hyperinsulinemia, and may increase oxidative stress, which inhibits the internalization and the actions of insulin (7).

Insulin deficiency, hepatic injury and spleen dysfunction are common pathological findings in patients with excess iron. Particularly, pancreatic β cells, the main cell types of Langerhans islets, compared with other cells is severely sensitive to the toxicity of oxidative stress due to its low antioxidative defense system (8). It has been stated that a disproportion between high level of oxidative stress and low capacity for eliminating ROS is the key reason of the vulnerability of β cells, thus leading to a serious insulin insufficiency (9). So far, various therapies are used as an advantageous therapeutic platform for T2D and organ injuries act by activating antioxidant pathways. Hence, identification of underlying cellular and molecular processes that are related to ROS-related tissue dysfunctions can be targeted for treatment of iron overload disorders in patients (10).

Based on these findings, preliminary studies have evaluated the effect of iron depletion by phlebotomy or chelators on iron-induced cell or tissue damage (11). Iron depletion reduced hyperinsulinemia, hyperglycemia, and improved liver function in patients with T2D and NAFLD. However, increased iron accumulation contributes to insulin resistance by

affecting redox status in hepatocytes (12). The current clinically available iron-chelating agents deferoxamine, deferiprone, and deferasirox show several side effects and limitations. Accordingly, a new avenue is required to provide more effective treatment with less side effects to patients with iron overload. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) (FA) is a plant phenolic acid that turns out to be a potential antioxidant phenoxyl radical (13). FA is commonly found in several herbs, especially in traditional Chinese medicine. It is easily available, inexpensive and exerts minimal side-effects. Phenolic compounds have protective roles on coronary heart diseases, cancer, neurodegenerative diseases, and inflammation as potential therapeutic agents (14). Due to the hydroxyl and phenoxy groups in FA, it is a strong antioxidant that can neutralise nitric oxide and hydroxyl radical groups and scavenge electrons. Thus, FA plays a role in the prevention of DNA damage, cell death and oxidative stress. Moreover, it is known that FA could promote functional recovery against oxidative stress through inducing the NRF2 antioxidant pathway (15).

Induction of antioxidant cellular defence is considered one of the important mechanisms to protect cells from oxidative injuries, such as the activation of the transcription factor NRF2 (nuclear factor erythroid 2-related factor 2). NRF2 plays a vital role in antioxidant defence mechanisms by regulating a battery of downstream detoxifying and antioxidant genes, such as NAD(P)H quinone oxidoreductase 1 (NQO1), haem oxygenase 1 (HO-1), cystine/glutamate antiporter (SLC7A11), Glutathione peroxidase 4 (GPX4), glutamate-cysteine ligase catalytic subunit (GCLC). Therefore, the proper function of NRF2 is essential for the survival of cells against oxidative stress-related damage by its antioxidant enzymes that maintain the intracellular redox status (16).

The liver is the major site of iron storage and pathologic iron accumulation. Production of ROS increases in the liver when its capacity to maintain iron in storage forms is exceeded. Therefore, elucidating the effect of phenolic acids such as FA and FAS on hepatic oxidative metabolism is critical for designing effective hepatic antioxidant therapies. In addition to *in vivo* studies, HepG2 were chosen as convenient models of cell membranes, and in the case of HepG2 as a model of liver iron overload (17). To better characterize the relationship between phenolic acids and excess iron, we next evaluated *in vitro* and *in vivo* whether phenolic acids

are able to interfere with iron related oxidative stress in HepG2 cells, liver tissues and other tissues.

To investigate the possible regulatory mechanisms that underlie the cytoprotective effects of FA, the present study examined the role of FA both against iron-induced toxicity in organs and HepG2 cells by activating the NRF2 pathway. In addition, alterations in both antioxidant mechanisms, such as MDA, GSH, SOD and iron related genes including DMT1, FPN and TfR1 were investigated. Furthermore, it was demonstrated that NRF2 has a central role in the protection of cells against iron toxicity through the upregulation of multiple antioxidant genes (NQO1, GCLC, HO-1, GPX4) involved in ROS metabolism. Collectively, these results indicate that FA can be a potent antioxidant against iron-induced injury by activating the NRF2 pathway.

5.2. Results

Effect of FA against iron-induced damage in HepG2 cells

Iron overload was induced with FAC and the lipophilic iron chelator 8HQ. FAC is a relatively stable formulation of ferric citrate, a species of free iron found in blood that increases in hereditary hemochromatosis and other conditions of iron overload (18). 8HQ facilitates rapid (within minutes) entry of iron into cells to transiently overload cellular defences against free iron (19). The cytotoxicity of rapid iron overload on HepG2 cells was evaluated using the MTT-cell viability assay. Cells were incubated with iron complex (50 μ M FAC-20 μ M 8HQ) for 2 h. 8HQ-mediated iron entry exhibited toxic effects in decrease of 48% cell viability compared with the control group (Figure 5.1). To investigate the protective effects of phenolic acids against 8HQ-mediated 50 μ M FAC, MTT assay was conducted to determine cell viability. HepG2 cells were treated with 5 different concentrations of FA or FAS at 5, 10, 20, 30 and 40 μ M for overnight and then exposed to 50 μ M FAC-20 μ M 8HQ for 2 h. This study shows that cell viability was significantly increased at 10 μ M FA or FAS after pretreatment of 50 μ M FAC-20 μ M 8HQ compared with the 50 μ M FAC-20 μ M 8HQ group (Figure 5.1A and 1B). Consequently, the effective concentrations of both phenolic acids at 10 μ M FA or FAS and 50 μ M FAC-20 μ M 8HQ were selective for further experiments.

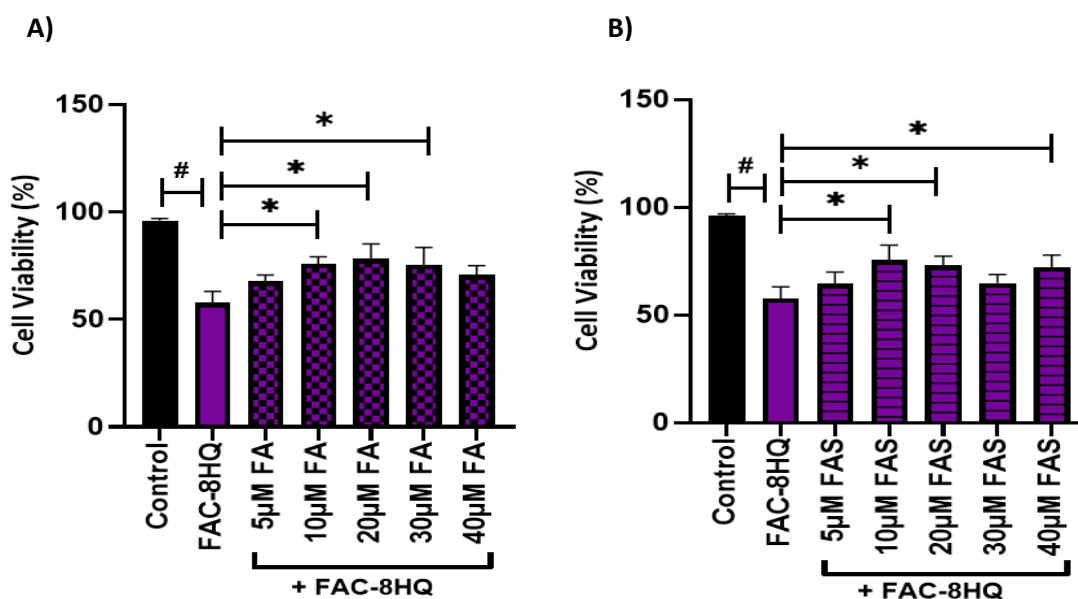


Figure 5.1. Determination of dose of phenolic acids to prevent iron-induced HepG2 cell damage. HepG2 cells were supplemented with (5, 10, 20, 30 and 40 μ M) ferulic acid (FA) or, (5, 10, 20, 30 and 40 μ M) ferulic acid 4-O-sulfate disodium salt (FAS) at the dose dependent manner for overnight. Then, cells were treated with 50 μ M ferric ammonium citrate and 20 μ M 8-hydroxyquinoline (FAC-8HQ) for 2 h to implement rapid iron entry into cells. Cell viability was assayed to show protective effects of FA or FAS against iron overload treatment. All the values are expressed with the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control vs. treatment groups, * $p < 0.05$ FAC-8HQ only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Reduced production of ROS and lipid peroxidation in iron treated HepG2 cells

HepG2 cells are suitable *in vitro* model system to investigate oxidative stress-related insulin resistance (20). To verify that phenolic acids can attenuate FAC-induced oxidative stress in HepG2 cells, ROS levels were measured by a DCFH-DA probe. As shown in Figure 5.2A, a significant increase of ROS levels was noted in HepG2 cells after treatment with 50 μ M FAC-20 μ M 8HQ for 2 h ($p < 0.05$). However, pre-treatment of HepG2 cells with 10 μ M FA or FAS for overnight suppressed the increase of ROS compared with the 8HQ-mediated iron treatment group. These results revealed that phenolic acids could attenuate the intracellular ROS accumulation ($p < 0.05$) in the cells.

Furthermore, ROS might react with lipids in cell and mitochondrial membranes, resulting in the production of MDA which is a biomarker of lipid peroxidation. Due to its strong cytotoxicity, MDA alters membrane permeability or disrupt membrane integrity, leading to oxidative injury. As shown in Figure 5.2B, 50 μ M FAC-20 μ M 8HQ treatment increased MDA

production compared with the control group ($p < 0.05$) whereas 10 μM FA or FAS pre-treatment of similarly treated HepG2 cells significantly decreased MDA production compared with the iron treatment group.

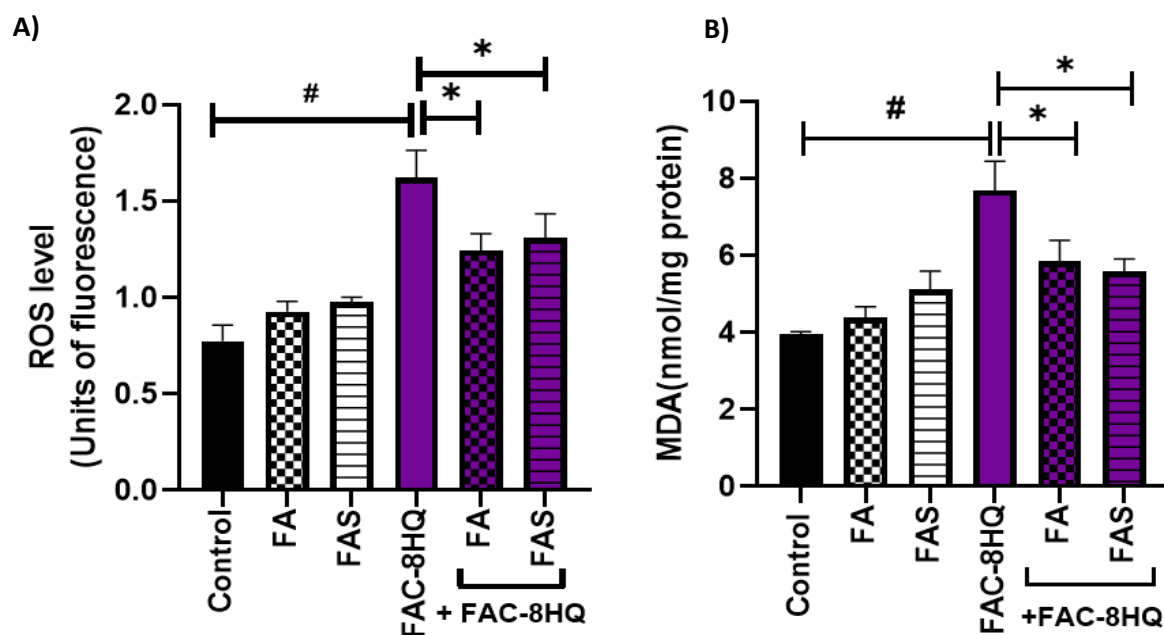


Figure 5.2. Protective effects of phenolic acids on HepG2 exposed to iron from intracellular reactive oxygen species (ROS) generation and lipid peroxidation (MDA). HepG2 cells were exposed to 20 μM ferulic acid (FA) or, 20 μM ferulic acid 4-O-sulfate disodium salt (FAS) for overnight. Then, cells were supplemented with 50 μM ferric ammonium citrate and 20 μM 8-hydroxyquinoline (FAC-8HQ) for 2 h to implement rapid iron entry into cells. Then, cellular reactive oxygen species (ROS) by dihydrodichlorofluorescein (H₂DCF) cell-permeant probe (A), lipid peroxidation levels by Malondialdehyde (MDA) assay (B) were measured. Data are presented as the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control group vs. treatment groups, * $p < 0.05$ vs. FAC-8HQ group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Increased levels of endogenous antioxidants in iron treated HepG2 cells

It was next determined whether FA or FAS pre-treatment can alleviate iron-induced oxidative stress by activating the endogenous antioxidant defence system. GSH is an important endogenous antioxidant, which reduces levels of ROS. Therefore, the level of GSH is an important factor in the determination of antioxidant capacity (21). Antioxidants and Fe are key regulators of oxidative stress. Fe can convert lipid peroxide into ROS, and GPX4 neutralizes iron-dependent lipid peroxidation by converting lipid hydroperoxides (L-OOH) to non-toxic lipid alcohols (L-OH) with GSH as a co-factor (22). As shown in Figure 3A, when compared with the control group, the level of GSH was significantly decreased ($p < 0.05$) with 50 μ M FAC-20 μ M 8HQ, however, 10 μ M FA and FAS pre-treatment increased GSH levels in HepG2 cells exposed to 50 μ M FAC-20 μ M 8HQ ($p < 0.05$).

SOD is a highly conserved enzyme, that is abundantly expressed in the cytoplasm of cells and plays a fundamental role in protecting cells from oxidative damage. Figure 5.3B showed that SOD levels were increased by FAS pre-incubation in HepG2 cells exposed to rapid iron entry compared with iron treated group.

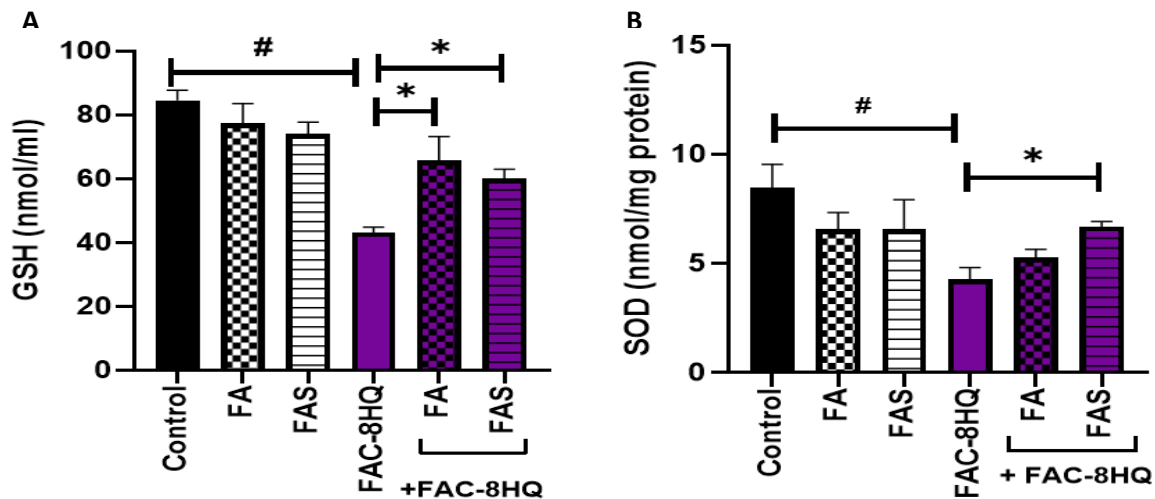


Figure 5.3. Effect of phenolic acids on the activity of antioxidant enzymes. HepG2 cells were exposed to 20 μ M ferulic acid (FA) or, 20 μ M ferulic acid 4-O-sulfate disodium salt (FAS) for overnight. Then, cells were supplemented with 50 μ M ferric ammonium citrate and 20 μ M 8-hydroxyquinoline (FAC-8HQ) for 2 h to implement rapid iron entry into cells. Then, glutathione (GSH) amounts by total GSH assay (A) and cellular superoxide dismutase amounts (SOD) (B) by SOD assay were measured. Data are presented as the mean $\pm$ SEM, $n = 8$. Experiments were repeated 3 independent times. # $p < 0.05$ control group vs. treatment groups, * $p < 0.05$ vs. FAC-8HQ group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Attenuation of tissue iron content and oxidative stress by phenolic acids

Condition of iron overload was elucidated by iron dextran injections in in vivo studies, that mimic parenteral administration of iron supplements. Iron dextran is a preparation of ferric hydroxide complexed with a low molecular weight fraction of dextran. It has been well established that after injection, elemental iron is then released from the complex by macrophages (mainly by Kupffer cells) and successively re-enters plasma via a ferroportin-dependent pathway which rapidly increases plasma iron levels (23).

Iron accumulation in the tissues was evaluated with the non-haem iron assay, to investigate if FA or FAS could influence the iron deposition levels in the liver, spleen, and kidney. Experimental groups subjected to iron overload in the liver, spleen and kidney exhibited increased levels of non-haem iron at almost 2-fold. However, treatments of phenolic acids, FA and FAS, appreciably reduced iron content in the liver, kidney (Figure 4). However, FAS treatment of iron overloaded mice decreased the amount of non-haem iron accumulation in spleen tissues ($p < 0.05$) (Figure 5.4B).

Perl's Prussian blue staining was used to detect the non-haem iron deposition in the liver, spleen. The blue staining is proportional to the amount of iron detected in the tissue. The microscopic images revealed the differences between the control and the iron dextran group in the liver (Figure 5.5). The staining in the spleen revealed some differences between the iron dextran group and the control group (Figure 5.6).

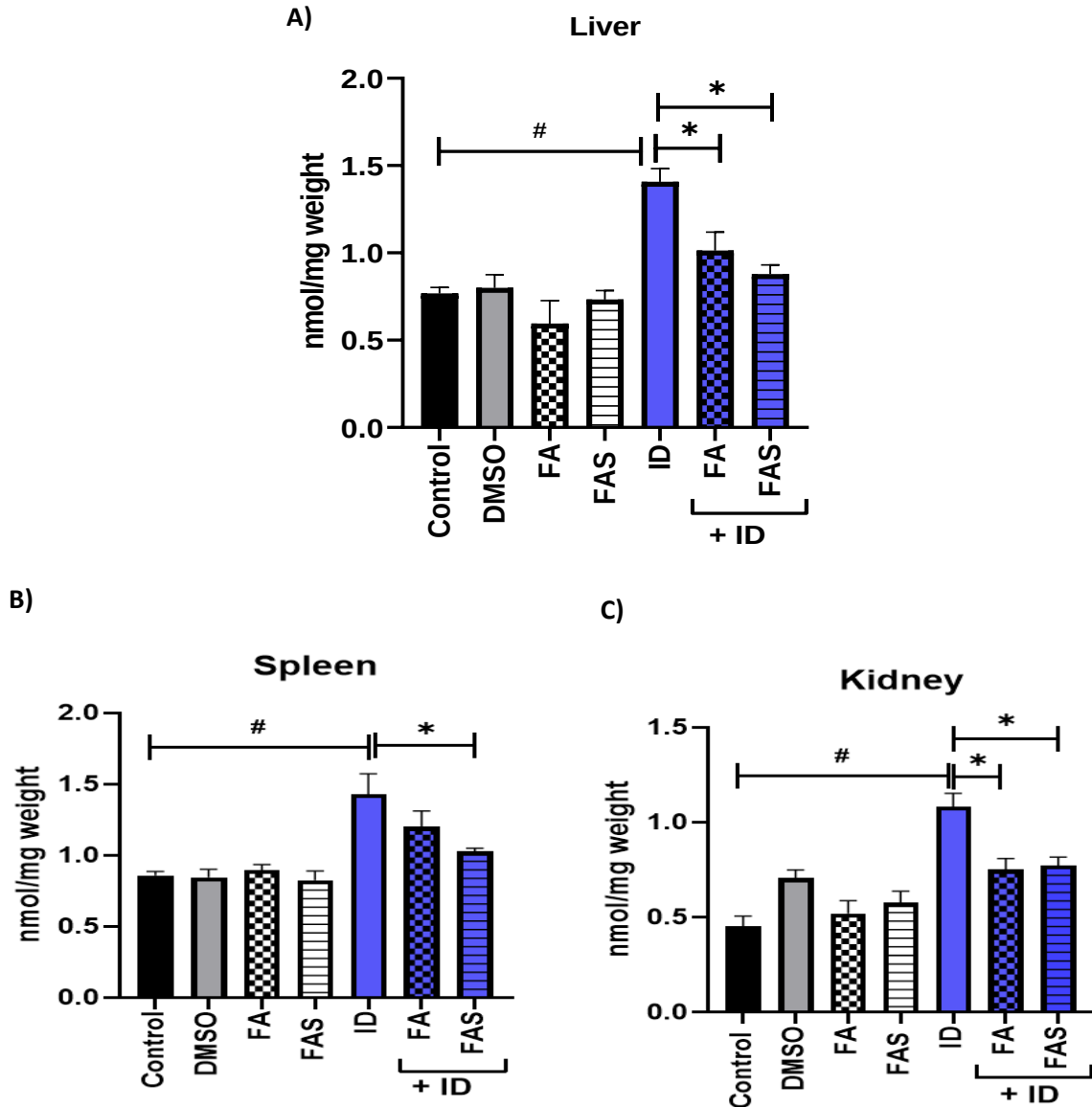
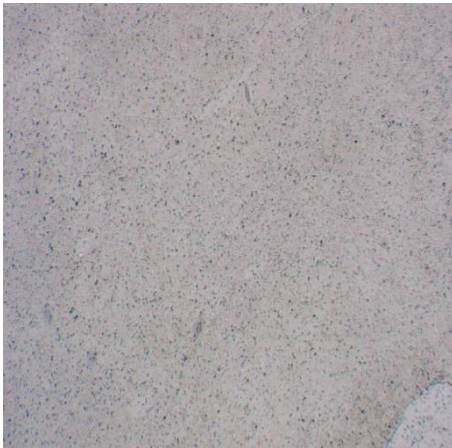


Figure 5.4. Non-haem iron levels in the tissues. BALB/c male mice were randomly divided into seven groups ($n=5$ mice per group), (1) Control, (2) Iron dextran (ID), (3) Ferulic acid (FA) and (4) Ferulic Acid 4-O-Sulfate Disodium Salt (FAS), (5) DMSO, (6) FA + ID, (7) FAS+ ID. ID group was injected intraperitoneally with iron dextran every two days 100 mg/per kg mice for 10 days. Mice were given orally FA or FAS 20 mg/per kg for 10 days by gavage, starting a day before the commencement of iron loading. The control group was given sterile saline by gavage for 10 days. Another group was administrated with 0.1% DMSO. Non-haem iron content in liver (A), spleen (B) and kidney (C) was measured calorimetrically using UV-VIS spectrophotometry. Data are presented as the mean $\pm$ SEM, $^{\#}p<0.05$ control group vs. treatment groups, $*p<0.05$ ID group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

A) Control



B) ID

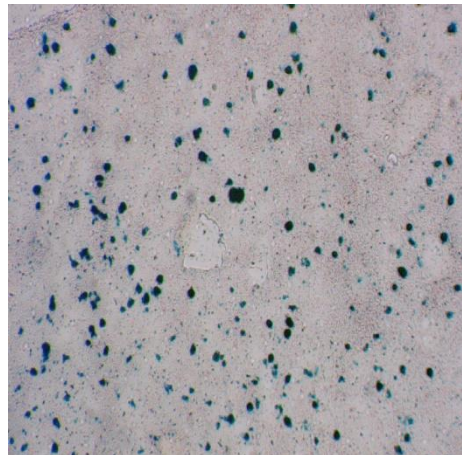
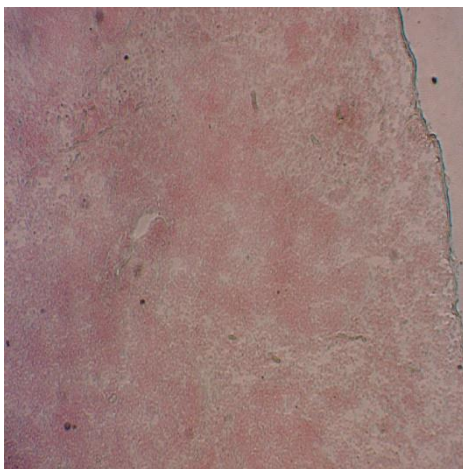


Figure 5.5. Perl's staining in the liver tissue of BALB/c mice. Immunohistochemistry of iron situations in liver of BALB/c mice showed in the control (saline-receiving mice) and iron dextran (100mg/kg) (ID) groups. Blue dots indicate iron deposition. Massive iron deposition in the liver tissues occurred in iron-overloaded mice compared with the normal saline-receiving mice. Observations were made in two slides per group.

A) Control



B) ID

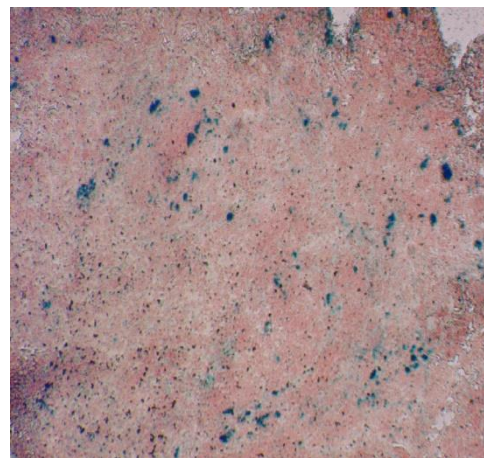


Figure 5.6. Perl's staining in the spleen tissue of BALB/c mice. Immunohistochemistry of iron situations in spleen of BALB/c mice showed in the control (saline-receiving mice) and iron dextran (100mg/kg) (ID) groups. Blue dots indicate iron deposition. Massive iron deposition in the spleen tissues occurred in iron-overloaded mice compared with the normal saline-receiving mice. Observations were made in two slides per group.

As a marker of tissue damage, lipid peroxidation was estimated from the levels of malondialdehyde (MDA) in the liver, spleen, and kidney. The MDA levels in the liver, spleen and kidney were highest in the group treated with iron dextran alone ($p < 0.05$) (Figure 5.7); however, FA or FAS treatments displayed a statistically significant decrease in the MDA levels in the liver and spleen, FA in kidney of mice ($p < 0.05$) (Figure 5.7).

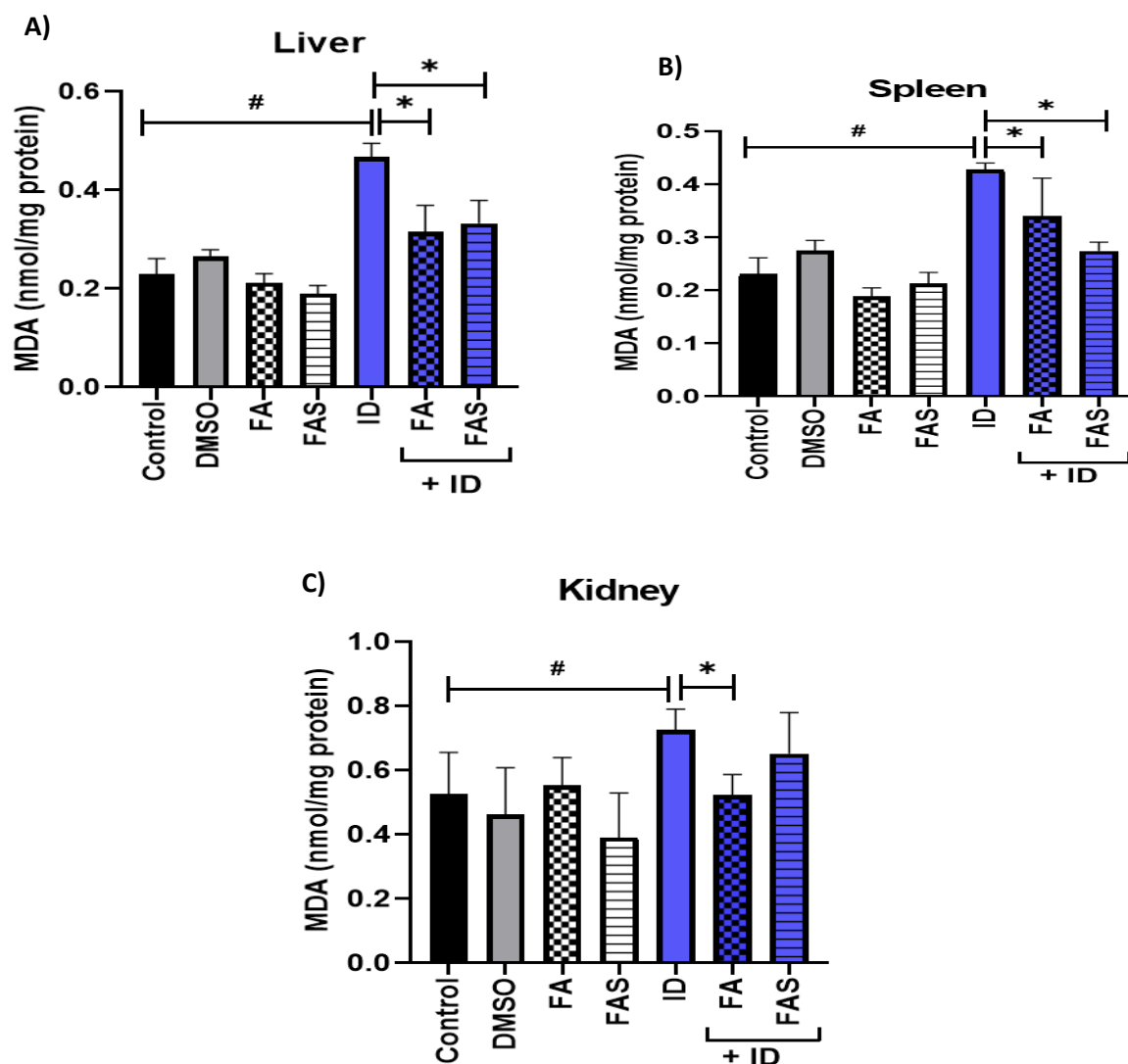


Figure 5.7. Malondialdehyde (MDA) levels in tissues of mice. BALB/c male mice were randomly divided into seven groups ($n=5$ mice per group), (1) Control, (2) Iron dextran (100 mg/kg)(ID), (3) Ferulic acid (20 mg/kg)(FA) and (4) Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS), (5) DMSO (0.1%), (6) FA + ID, (7) FAS+ ID. Lipid peroxidation levels in liver (A), spleen (B) and kidney (C) was measured by MDA assay. MDA levels in the tissues of only iron dextran treatment were significantly different from the control. Phenolic acids on MDA levels attenuated effects of excess iron in liver (A), spleen (B) and kidney (C) of BALB/c mice exposed to ID. Data are presented as the mean $\pm$ SEM, # $p < 0.05$ control group vs. treatment groups, * $p < 0.05$ ID group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

Enhancement of antioxidant capacity in tissues

Free radicals have extremely short half-lives so that in most cases oxidative stress is evaluated by measuring the endogenous antioxidant defences such as glutathione (GSH), and superoxide dismutase (SOD) (24). Antioxidant capacities were evaluated by measuring the levels of GSH (Figure 5.8) and SOD (Figures 5.9), in the liver, spleen and kidney tissues.

In the liver and spleen, levels of reduced glutathione (GSH) were increased with the treatment of FA or FAS in iron overloaded mice compared with only iron overloaded mice (Figure 5.8A and 8B). However, mice administered FAS treatment and iron dextran significantly increased kidney GSH levels compared with iron dextran only treated group (Figure 5.8C).

The levels of CuZn-SOD were measured in the liver, spleen, and kidney. It showed that iron dextran treatment group showed a decreased SOD activity compared with the control group in the tissues (Figure 5.9). In general, FA administration in iron dextran groups significantly recovered CuZn-SOD levels compared with iron dextran only groups of liver, spleen, and kidney tissues. Also, FAS treatments of iron overloaded mice showed a similar trend in spleen and kidney tissues; except for in the liver (Figure 5.9).

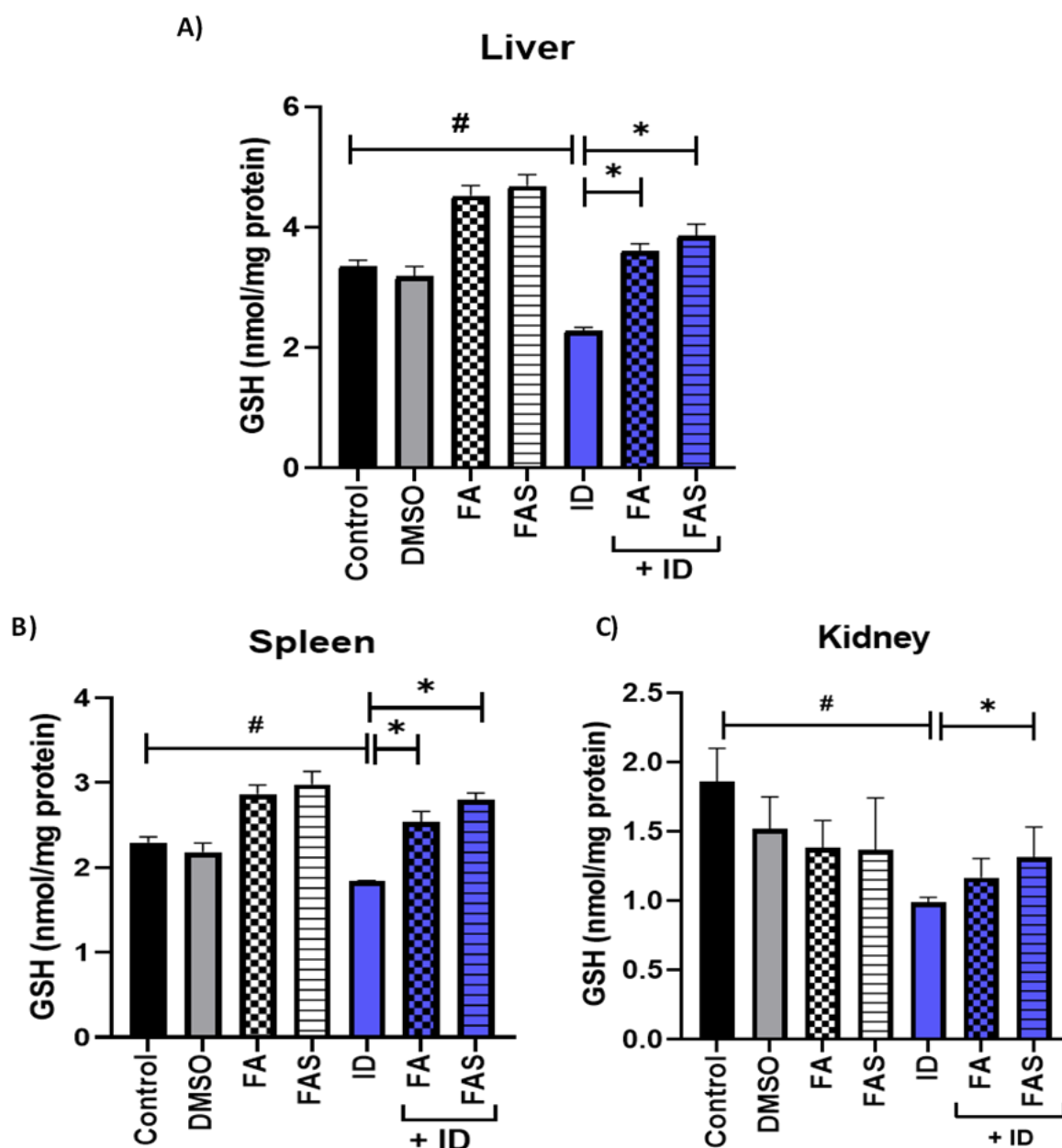


Figure 5.8. Glutathione (GSH) activity levels in the tissues of mice. BALB/c male mice were randomly divided into seven groups ($n=5$ mice per group), (1) Control, (2) Iron dextran (100 mg/kg)(ID), (3) Ferulic acid (20 mg/kg)(FA) and (4) Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS), (5) DMSO (0.1%), (6) FA + ID, (7) FAS+ ID. Antioxidant total glutathione amounts in liver (A), spleen (B) and kidney (C) was measured by GSH assay. GSH levels in the tissues of only iron dextran treatment were significantly different from the control. Phenolic acids on GSH levels moderated effects of excess iron in liver (A), spleen (B) and kidney (C) of BALB/c mice treated with ID. Data are presented as the mean $\pm$ SEM, $^{\#}p<0.05$ control group vs. treatment groups, $*p<0.05$ ID group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

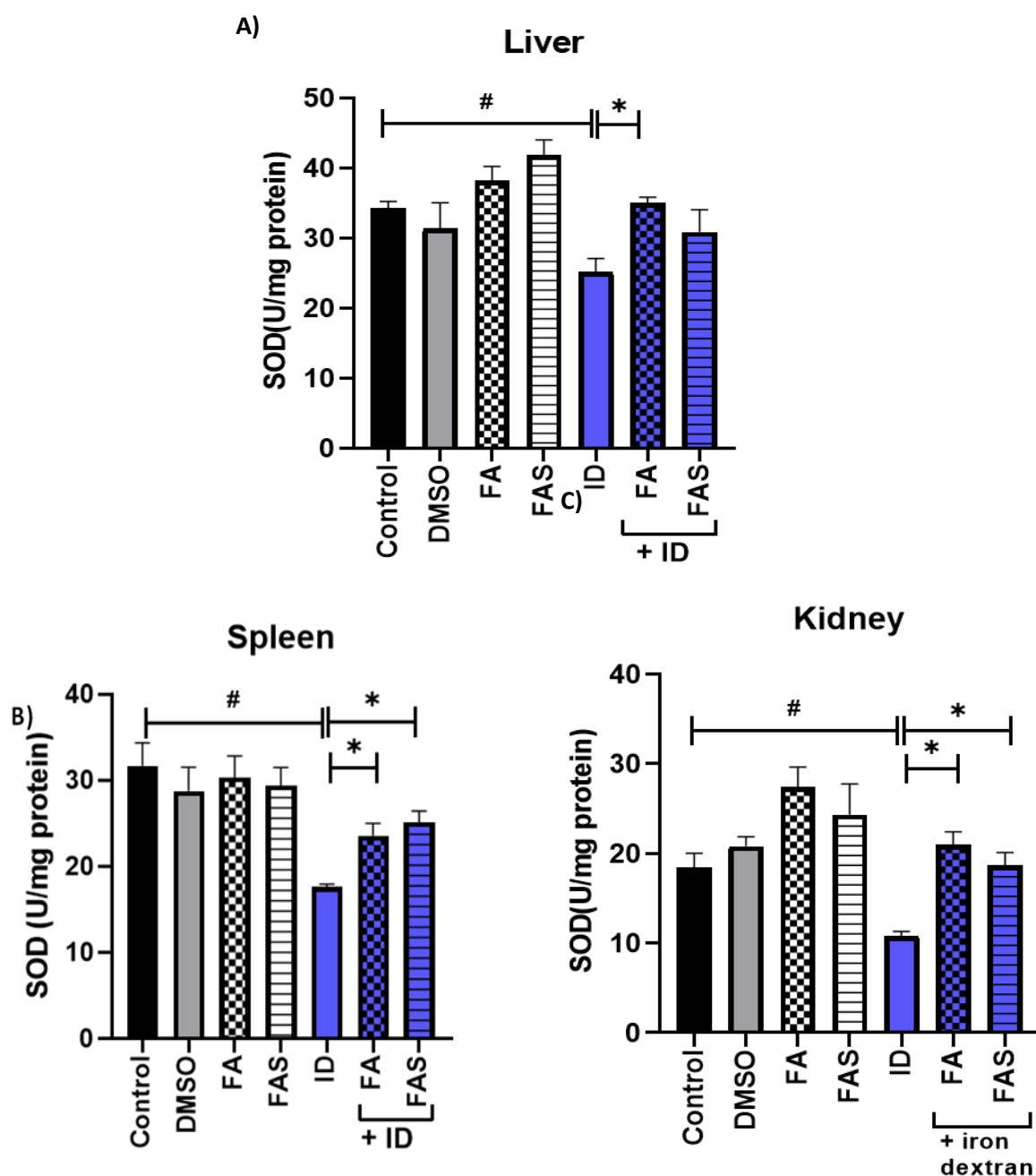
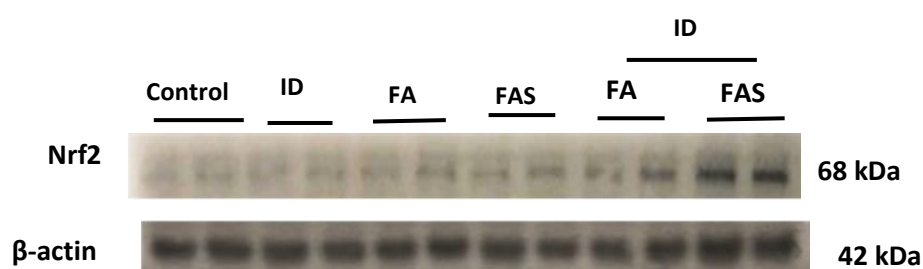


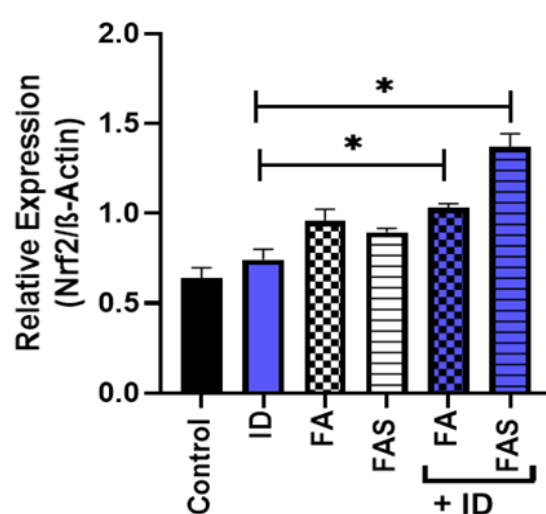
Figure 5.9. Zn-Cu superoxide dismutase (SOD) activity levels in the tissues of mice. BALB/c male mice were randomly divided into seven groups (n=5 mice per group), (1) Control, (2) Iron dextran (100 mg/kg)(ID), (3) Ferulic acid (20 mg/kg)(FA) and (4) Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS), (5) DMSO (0.1%), (6) FA + ID, (7) FAS+ ID. Zn-Cu superoxide dismutase (SOD) activity levels in liver (A), spleen (B) and kidney (C) was measured by Zn-Cu superoxide dismutase assay. SOD activity in the tissues of only iron dextran treatment were significantly different from the control. Phenolic acids on SOD levels alleviated effects of excess iron in liver (A), spleen (B) and kidney (C) of BALB/c mice treated with ID. Values are presented as the mean $\pm$ SEM, # $p < 0.05$ control group vs. treatment groups, * $p < 0.05$ ID group only vs. treatment groups. One-way ANOVA, Tukey post-hoc test.

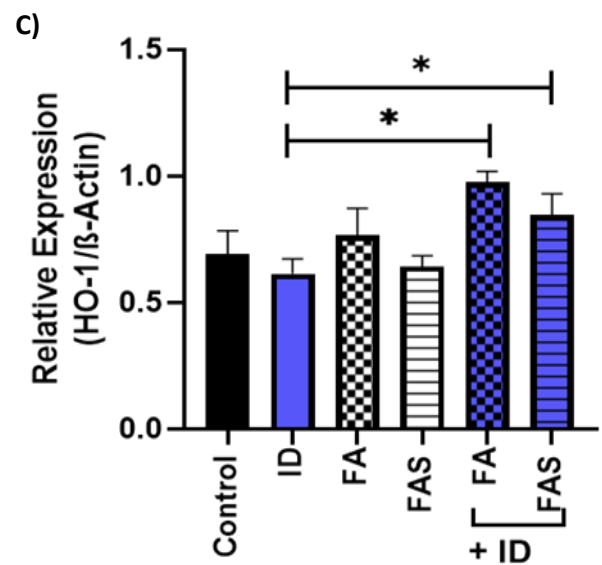
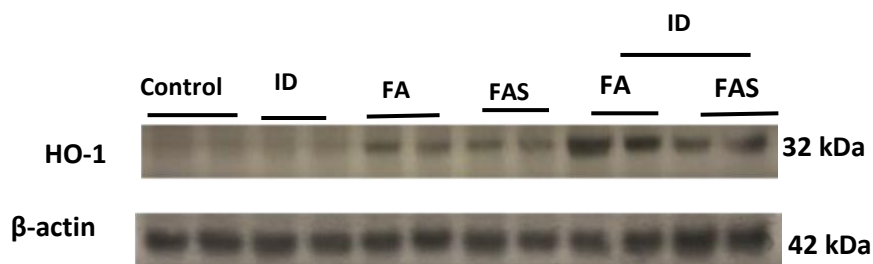
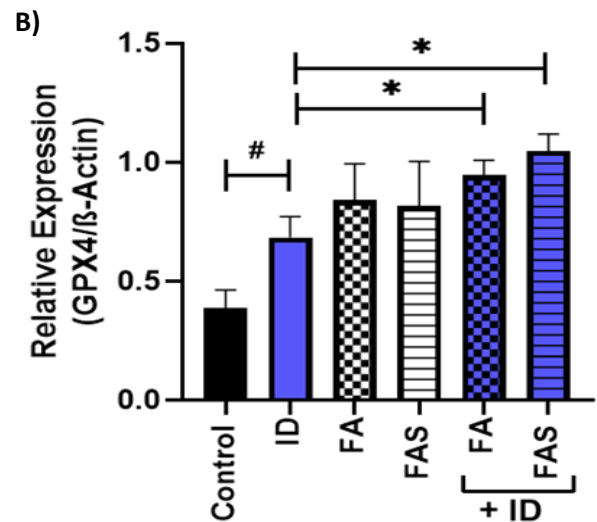
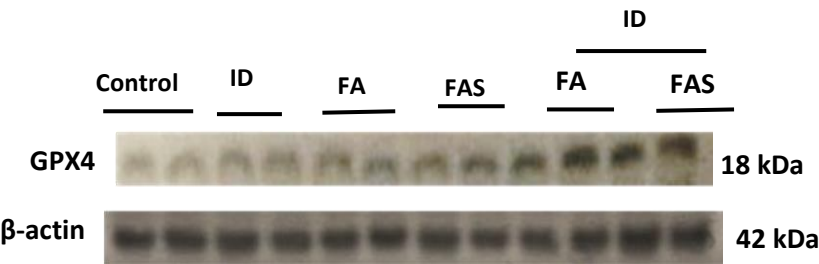
Effects of phenolic acids on antioxidant pathway in liver and pancreas

To further identify whether the protective effect of phenolic acids on tissues due to iron-induced oxidative stress is dependent on Nrf2 pathway, its downstream genes and proteins were detected in tissues treated with iron dextran by qPCR and Western blot respectively. As is illustrated in Figure 5.10, the protein levels of total Nrf2 and its downstream antioxidative genes including NQO1, GCLC, HO-1 and GPX4 were significantly increased in iron overloaded liver after treatments of phenolic acids. It has been further performed qPCR assays to partially confirm the results of Western blot analysis (Figure 5.11). Consistent with the changes of protein levels in the liver, the mRNA expressions of total Nrf2 and GPX4 in phenolic acid-treated groups in iron dextran loaded tissues were significantly elevated ($p < 0.05$) (Figure 5.12).



A)





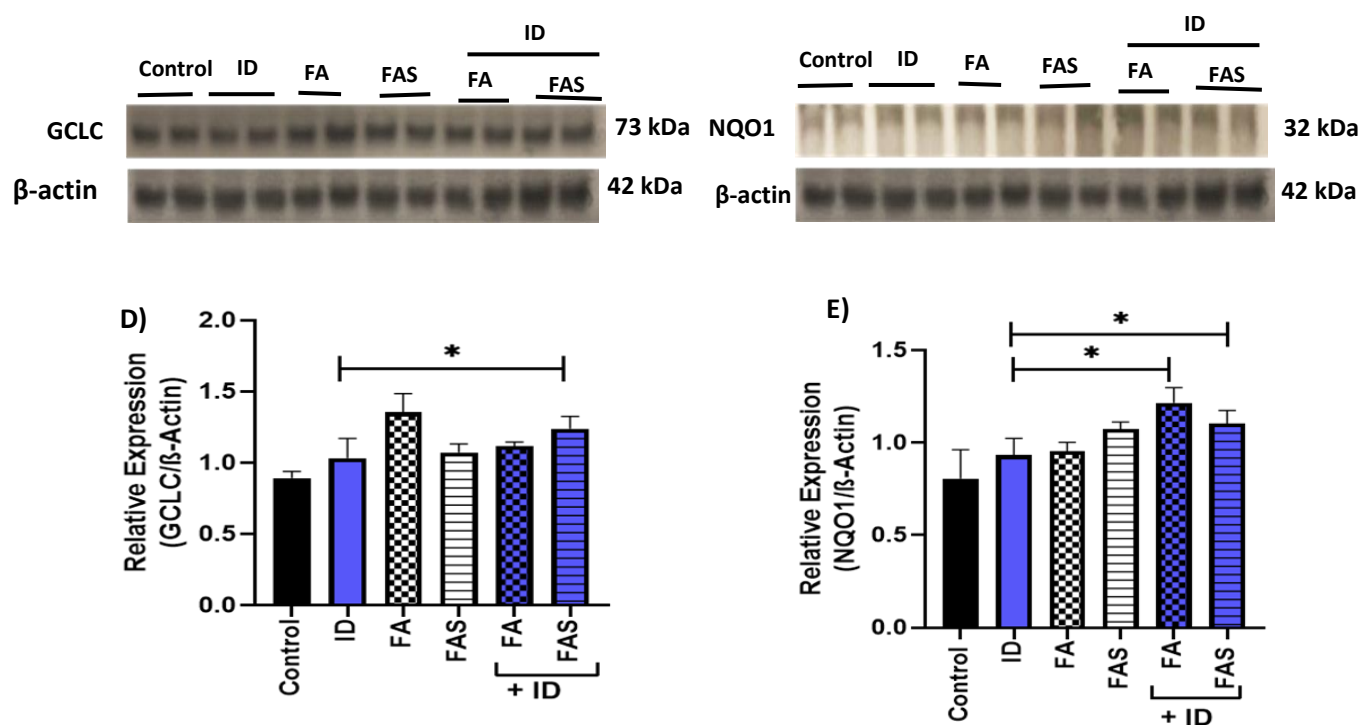


Figure 5.10. Expressions of proteins in Nrf2 antioxidant pathways. BALB/c male mice were randomly divided into seven groups ($n=5$ mice per group), (1) Control, (2) Iron dextran (100 mg/kg)(ID), (3) Ferulic acid (20 mg/kg)(FA) and (4) Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS), (5) DMSO (0.1%), (6) FA + ID, (7) FAS+ ID. Total proteins were extracted from liver tissues and immunoblot analyses for nuclear factor erythroid 2–related factor 2, Nrf2 (A), glutathione peroxidase 4, GPX4 (B), haem oxygenase-1, HO-1 (C), glutamate-cysteine ligase catalytic subunit, GCLC (D), NAD(P)H Quinone Dehydrogenase 1, NQO1 (E) were performed in liver tissues of BALB/c mice. Antioxidant protein expressions in the liver tissues of only iron dextran treatment were significantly different from the treatment groups of phenolic acids administrated with ID. Densitometry analysis of the blots was analysed with ImageJ software. The representative figure shows one of the two independent experiments. Data are presented as the mean $\pm$ SEM, $^{\#}p < 0.05$ control group vs. treatment groups, $^{*}p < 0.05$ vs. ID group only. One-way ANOVA, Tukey post-hoc test.

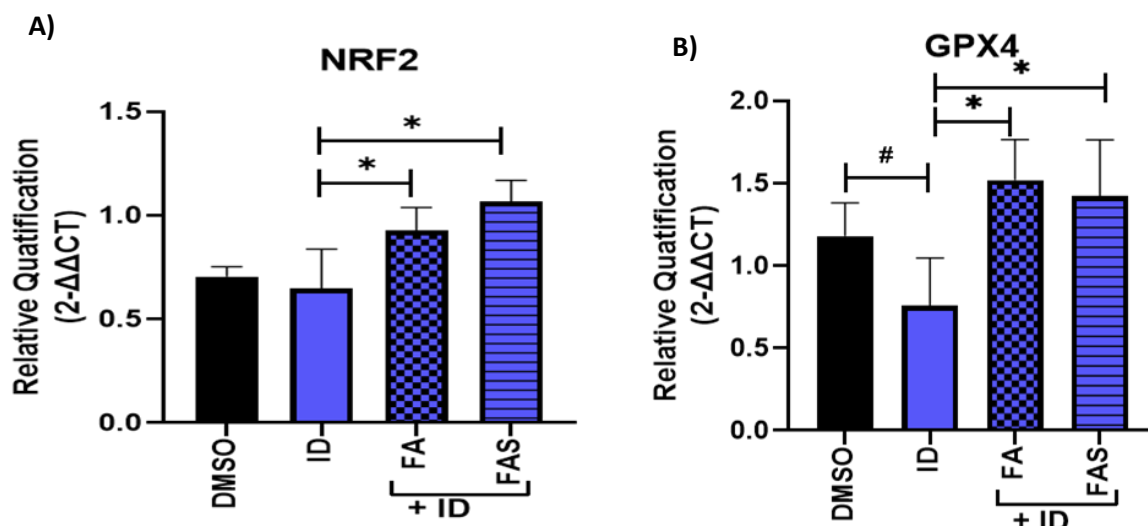
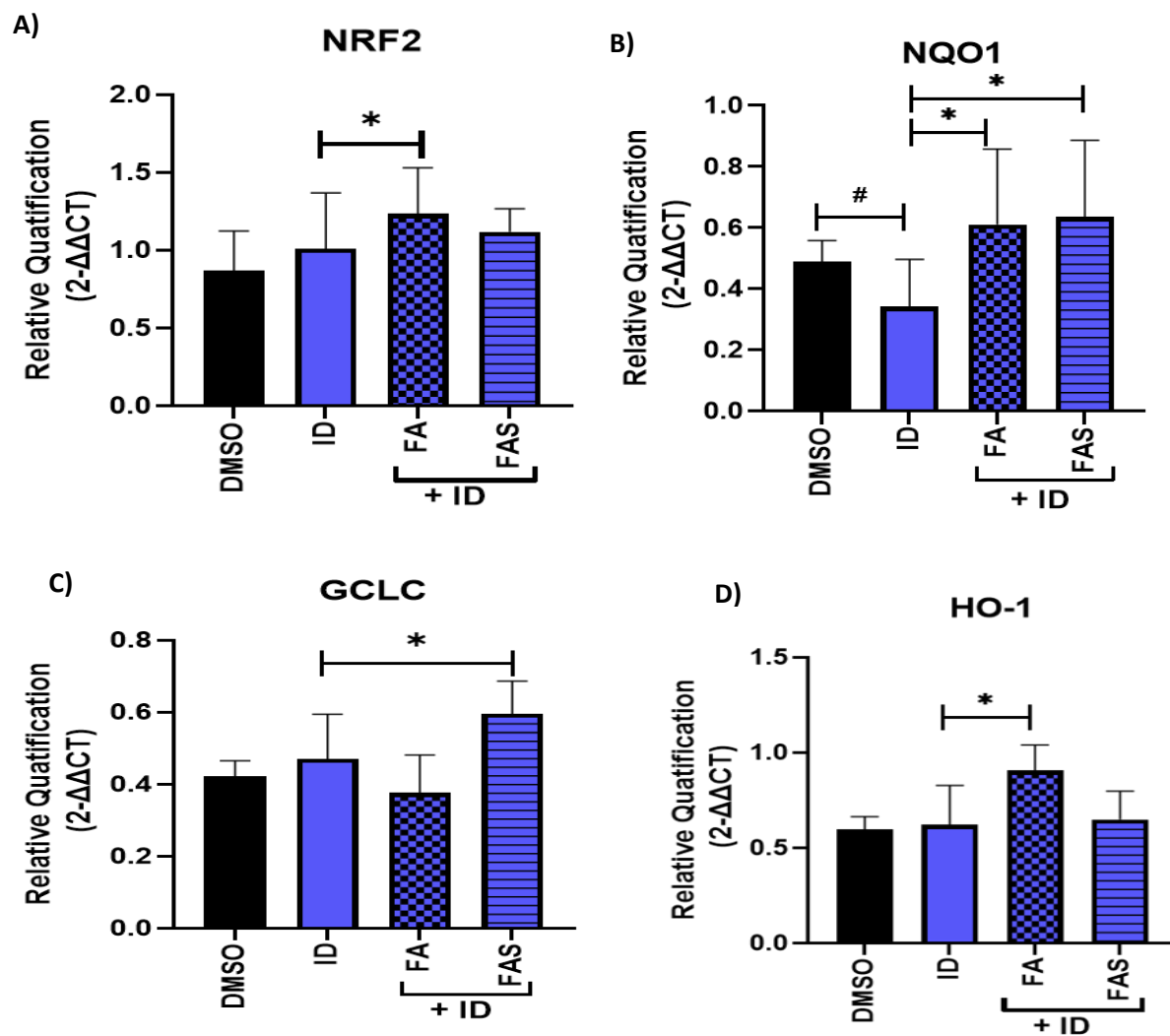


Figure 5.11. Expressions of *Nrf2* antioxidant pathway-related mRNA in the liver. The mRNA levels of nuclear factor erythroid 2-related factor 2, *Nrf2* (A), glutathione peroxidase 4, *GPX4* (B) were semi-quantified by RT-PCR analyses in liver tissues of BALB/c male mice exposed to DMSO (0.1%), Iron dextran (100 mg/kg)(ID), Ferulic acid (20 mg/kg)(FA) or Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS) with ID. Administrations of phenolic acids in mice exposed to ID increased the mRNA expressions of *Nrf-2* and *GPX4* compared with only ID group. Determination of gene expression by qPCR $2^{-\Delta\Delta CT}$ in the liver and normalized with which housekeeping gene. Data are presented as the mean $\pm$ SEM, $n = 5$ mice per group. # $p < 0.05$ DMSO vs. treatment groups, * $p < 0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.

The pancreas is a highly active organ especially in glucose metabolism, but its antioxidant capacity is low; however, the pancreas inherently contains only a fraction of the antioxidant enzymatic activities, in comparison to other organs (25). To next identify whether phenolic acids can show a protection in the pancreas under iron-induced oxidative stress by activating *Nrf2* pathway, mRNA levels of *Nrf2* and its downstream genes were detected in pancreas of mice exposed to iron dextran by qPCR. mRNA levels of *Nrf2*, *NQO1*, *GCLC*, *HO-1* and *GPX4* were increased significantly in the pancreas of iron-loaded mice that were treated with FA when compared with iron dextran treated only group (Figure 5.12). Also, FA treatment of iron dextran treated mouse pancreas showed significant increase in *NQO1*, *GCLC* and *GPX4* compared with pancreas of mice treated with iron dextran only (Figure 5.12).



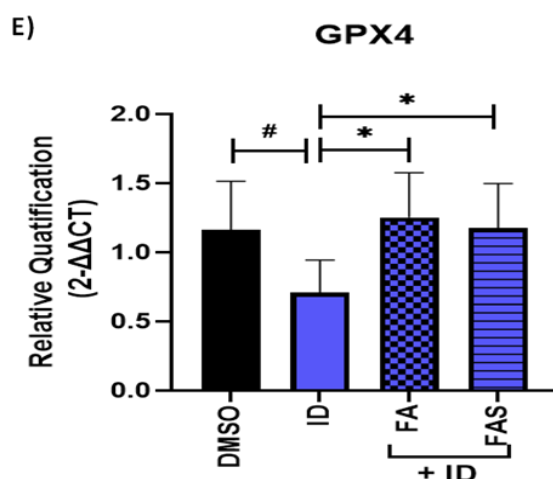
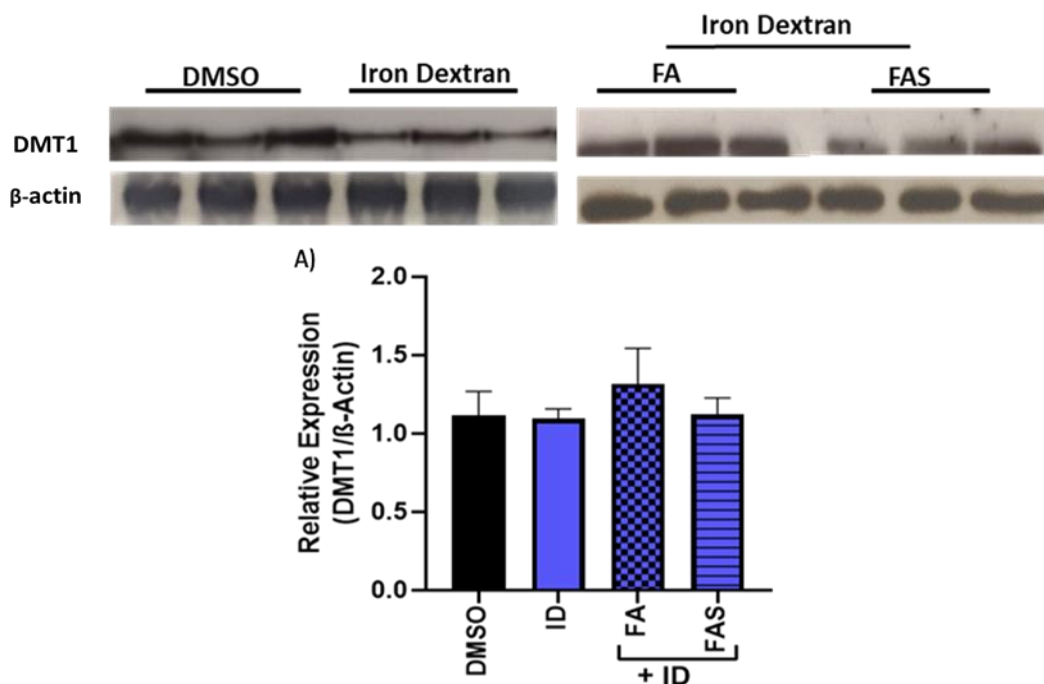


Figure 5.12. Expressions of Nrf2 antioxidant pathway-related genes in the pancreas. The mRNA levels of nuclear factor erythroid 2–related factor 2, Nrf2 (A), NAD(P)H Quinone Dehydrogenase 1, NQO1 (B), glutamate-cysteine ligase catalytic subunit, GCLC (C), haem oxygenase-1, HO-1 (D), glutathione peroxidase 4, GPX4 (E), were semi-quantified by RT-PCR analyses in pancreas tissues of BALB/c male mice exposed to DMSO (0.1%), Iron dextran (100 mg/kg)(ID), Ferulic acid (20 mg/kg)(FA) or Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS) with ID. Administrations of phenolic acids in mice exposed to ID increased the mRNA expressions of Nrf-2 and its downstream related genes compared with only ID group. Determination of gene expression by qPCR $2^{-\Delta\Delta CT}$ in the pancreas and normalized with which housekeeping gene. Data are presented as the mean $\pm$ SEM, $n = 5$ mice per group. # $p < 0.05$ DMSO vs. treatment groups, * $p < 0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.

Effects of phenolic acids on iron metabolism in liver and pancreas

The expression of ferritin, transferrin receptor-1 (*Tfr1*), divalent metal transporter-1 (DMT1), and ferroportin (*FPN*) mRNA by qPCR and protein by western blot were measured in the liver and pancreas tissues of the mice (Figure 5.13, 14 and 15). In general, the groups treated with iron dextran presented more changes in the expression of the iron transporters, however, among these groups the treatment with FA or FAS gave some differences.

The quantification of protein expression on iron metabolism by the Western blot was performed to confirm the results found in the qPCR assay. TfR1 increased significantly in the iron overloaded liver tissues of the group treated with FAS in comparison with the group treated with iron dextran only ($p < 0.05$) (Figure 5.13C). However, mRNA and protein levels of DMT1 did not show any significant difference in the liver tissues. FPN presented a marginal increase of mRNA and protein levels in the iron overloaded groups treated with both phenolic acids in comparison with the liver tissues of iron dextran only ($p < 0.05$) (Figure 5.13 and 14). The mRNA levels of ferritin light and heavy chain of liver samples increased significantly ($p < 0.05$) in the iron dextran group in comparison with the control group (Figure 5.14D and 14E). FAS treatment of iron dextran treated mice caused a decrease in mRNA levels of ferritin heavy chain unlike decreased the mRNA levels of ferritin light chain that was affected by FA in iron dextran treated mice compared with iron dextran only group ($p < 0.05$) (Figure 5.14).



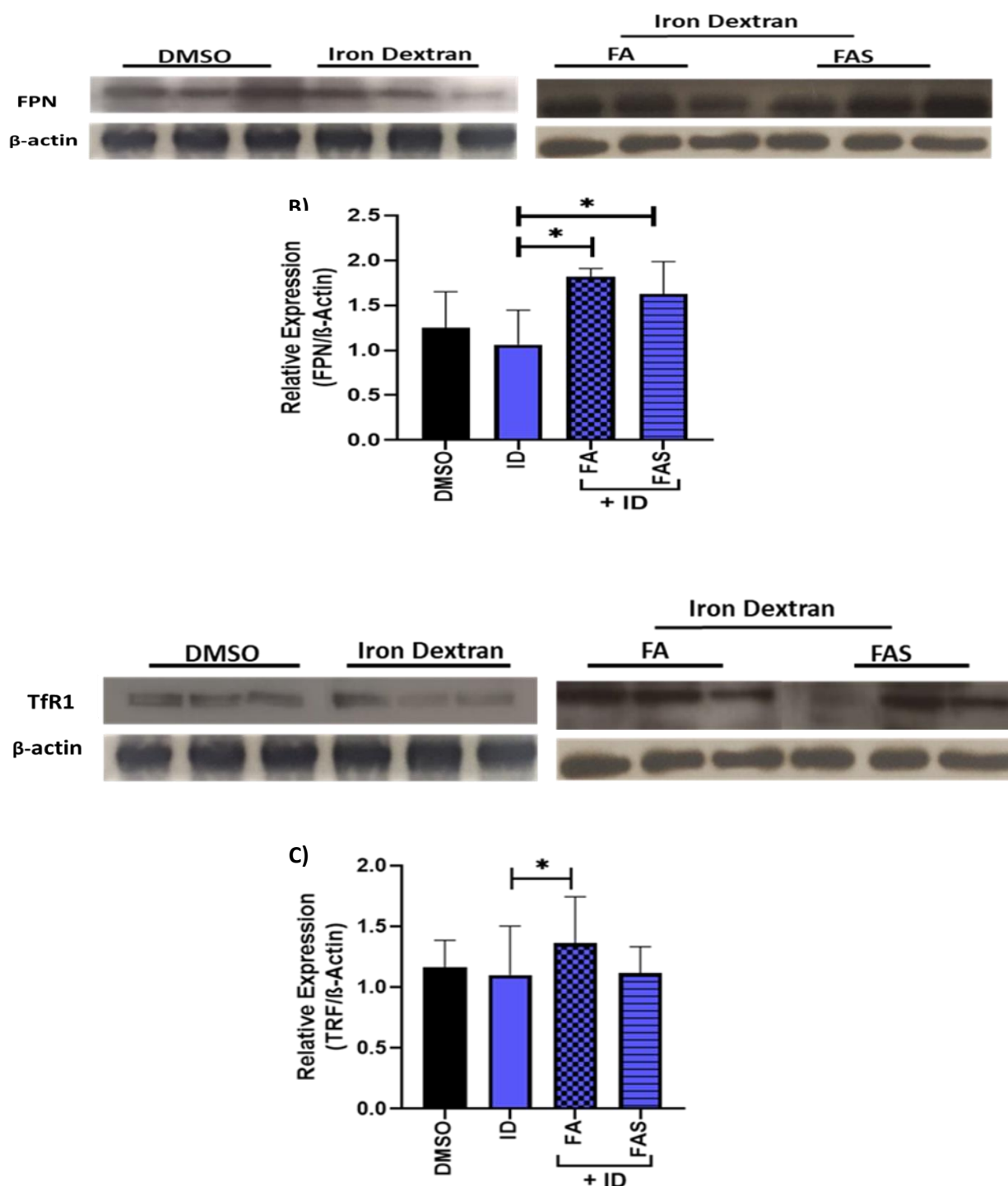
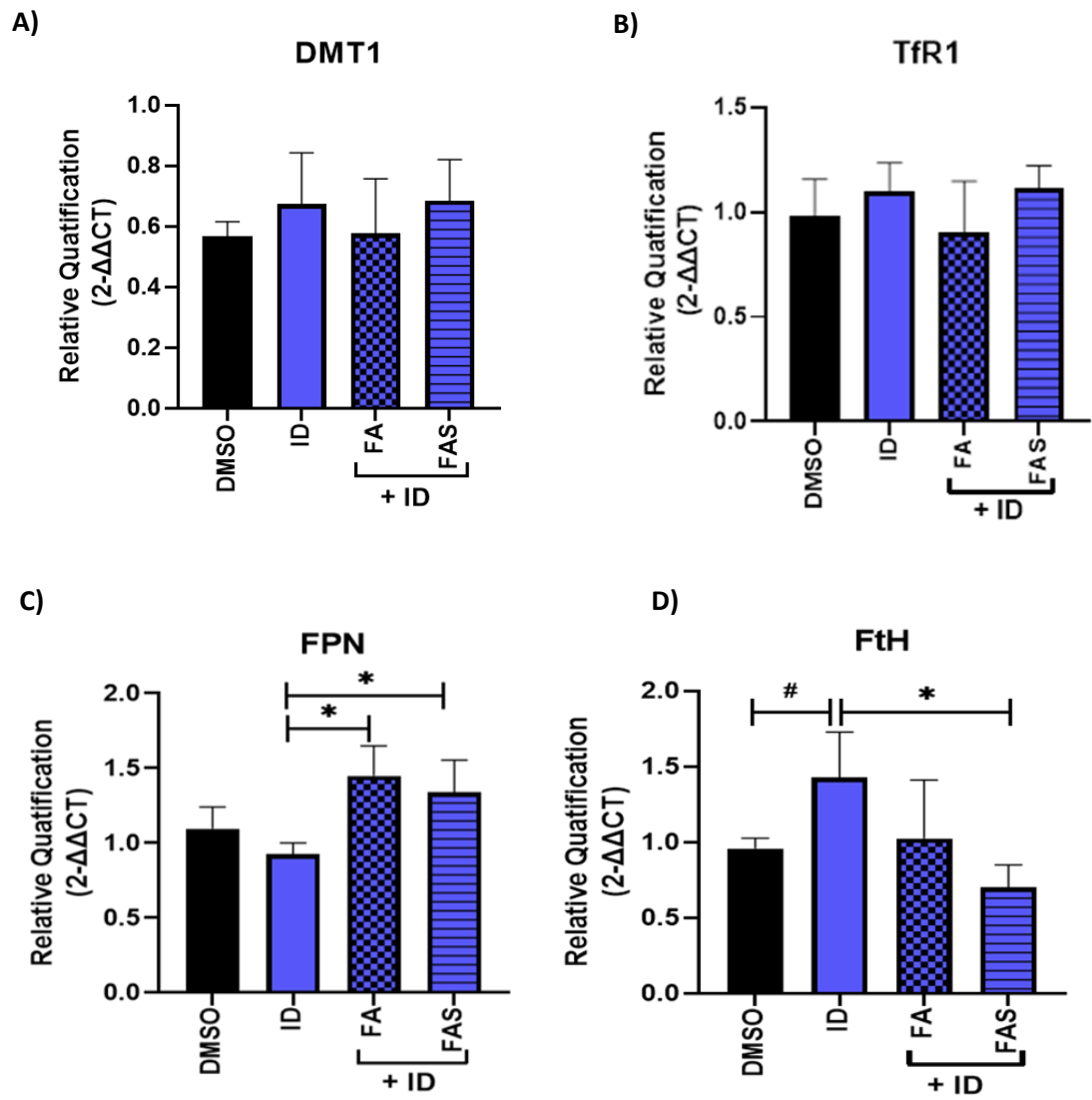


Figure 5.13. Protein expression of iron transporters in the liver. Total tissue lysates were prepared and subjected to Western blot analysis to monitor the expression levels of iron-related proteins including divalent metal transporter, DMT1 (A), ferroportin, FPN (B), transferrin receptor-1, TfR1 (C) in liver tissues of BALB/c male mice exposed to DMSO (0.1%), Iron dextran (100 mg/kg)(ID), Ferulic acid (20 mg/kg)(FA) or Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS) with ID. Actin served as an internal control. Administrations of phenolic acids in mice exposed to ID significantly increased protein expressions of FPN compared with only ID group. Densitometry analysis of the blots analysed with Image J software Data are presented as the mean $\pm$ SEM, $n = 3$. * $p < 0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.



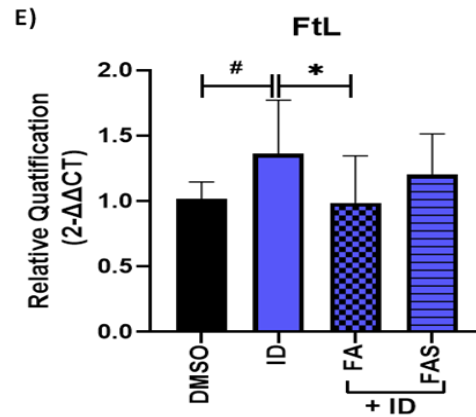


Figure 5.14. mRNA expression of iron transporters in the liver. The mRNA levels of divalent metal transporter, DMT1 (A), transferrin receptor-1, TfR1 (B), ferroportin, FPN (C), Ferritin heavy chain, FtH (D), Ferritin light chain, FtL (E) were semi-quantified by RT-PCR analyses in liver tissues of BALB/c male mice exposed to DMSO (0.1%), Iron dextran (100 mg/kg)(ID), Ferulic acid (20 mg/kg)(FA) or Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS) with ID. Administrations of phenolic acids in mice exposed to ID increased the mRNA expressions of FPN compared with only ID group. Determination of gene expression by qPCR $2^{-\Delta\Delta CT}$ in the liver and normalized with which housekeeping gene. Data are presented as the mean $\pm$ SEM, $n = 5$ mice per group. # $p < 0.05$ DMSO vs. treatment groups, * $p < 0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.

Expression of mRNA of the iron transporters was also determined in the pancreas of the mice that were treated with iron dextran and or with FA or FAS. Figure 5.15 shows the relative quantification of transferrin receptor-1 (*Tfr1*), ferroportin (*FPN*), ferritin light-chain (*FtL*), and ferritin heavy-chain (*FtH*). FA or FAS treatment of iron loaded mice displayed an increased trend of FPN mRNA levels compared with iron dextran treated only groups. For iron loaded mice treated with both phenolic acids, mRNA TfR1 and FtL levels were not different (Figure 5.15A and 15D). However, FA treatment caused significant decrease of FtH expression in the pancreas of iron-loaded mice ($p < 0.05$) (Figure 5.15C).

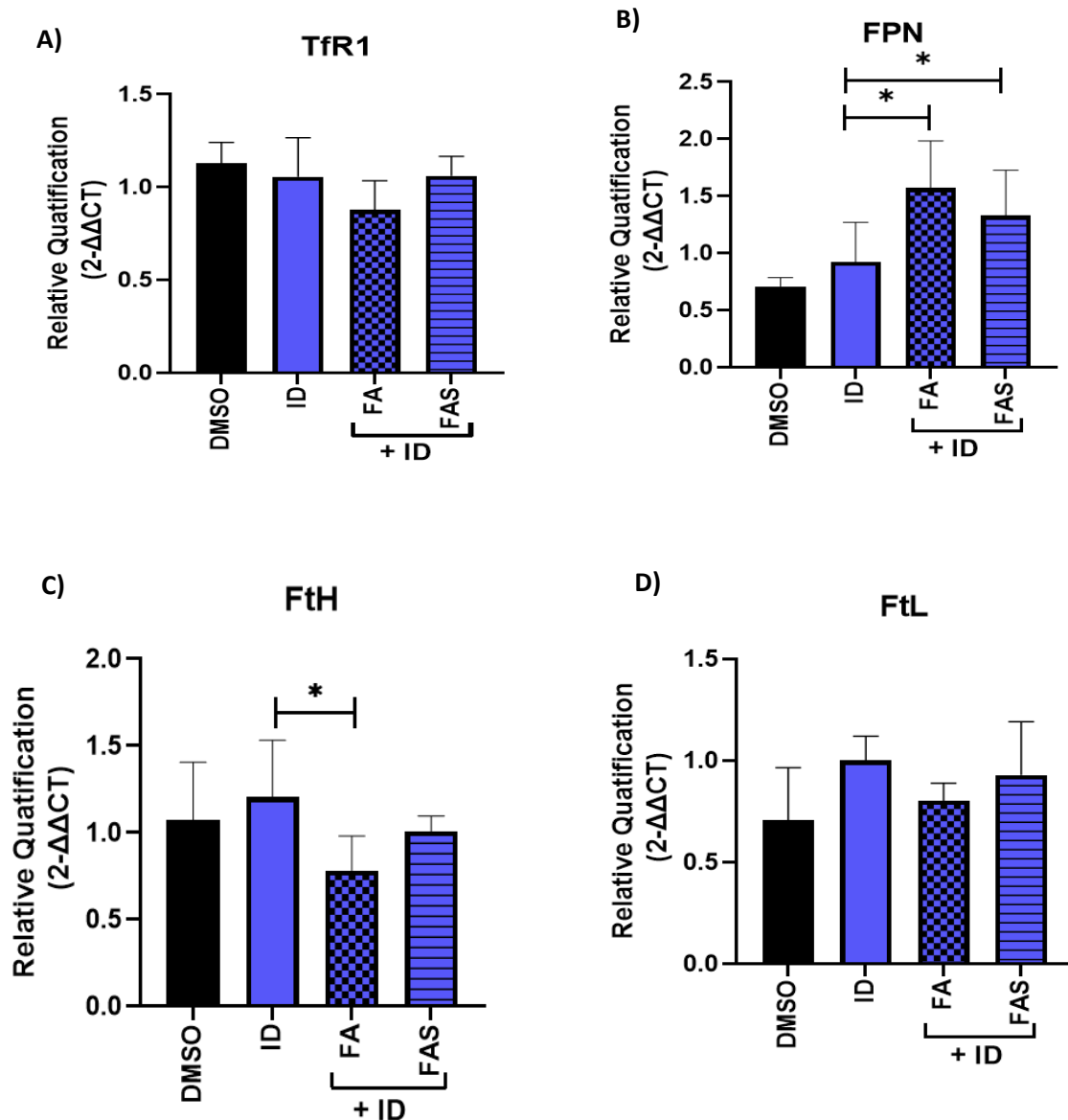


Figure 5.15. mRNA expression of iron transporters in the pancreas. The mRNA levels of transferrin receptor-1, TfR1 (A), ferroportin, FPN (B), Ferritin heavy chain, FtH (C), Ferritin light chain, FtL (D) were semi-quantified by RT-PCR analyses in pancreas tissues of BALB/c male mice exposed to DMSO (0.1%), Iron dextran (100 mg/kg)(ID), Ferulic acid (20 mg/kg)(FA) or Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS) with ID. Administrations of phenolic acids in mice exposed to ID increased the mRNA expressions of FPN compared with only ID group. Determination of gene expression by qPCR $2^{-\Delta\Delta CT}$ in the pancreas and normalized with which housekeeping gene. Data are presented as the mean $\pm$ SEM, $n = 5$ mice per group. # $p < 0.05$ DMSO vs. treatment groups, * $p < 0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.

The impact of phenolic acids on the mRNA expression of STAT3, SOCS3 and Foxo1

Furthermore, expression of mRNA of the inflammation transporters was measured in the liver. Figure 5.16 shows the relative quantification of signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signalling 3 (SOCS3) and Forkhead box protein O1 (Foxo1). ID treatment groups significantly ($p < 0.05$) increased the gene expressions stress related genes; STAT3 and Foxo1. FA treatment groups exposed the ID significantly ($p < 0.05$) inhibited the mRNA gene expressions STAT3, SOCS3 and Foxo1. However, FAS did not show any significant effect on those genes.

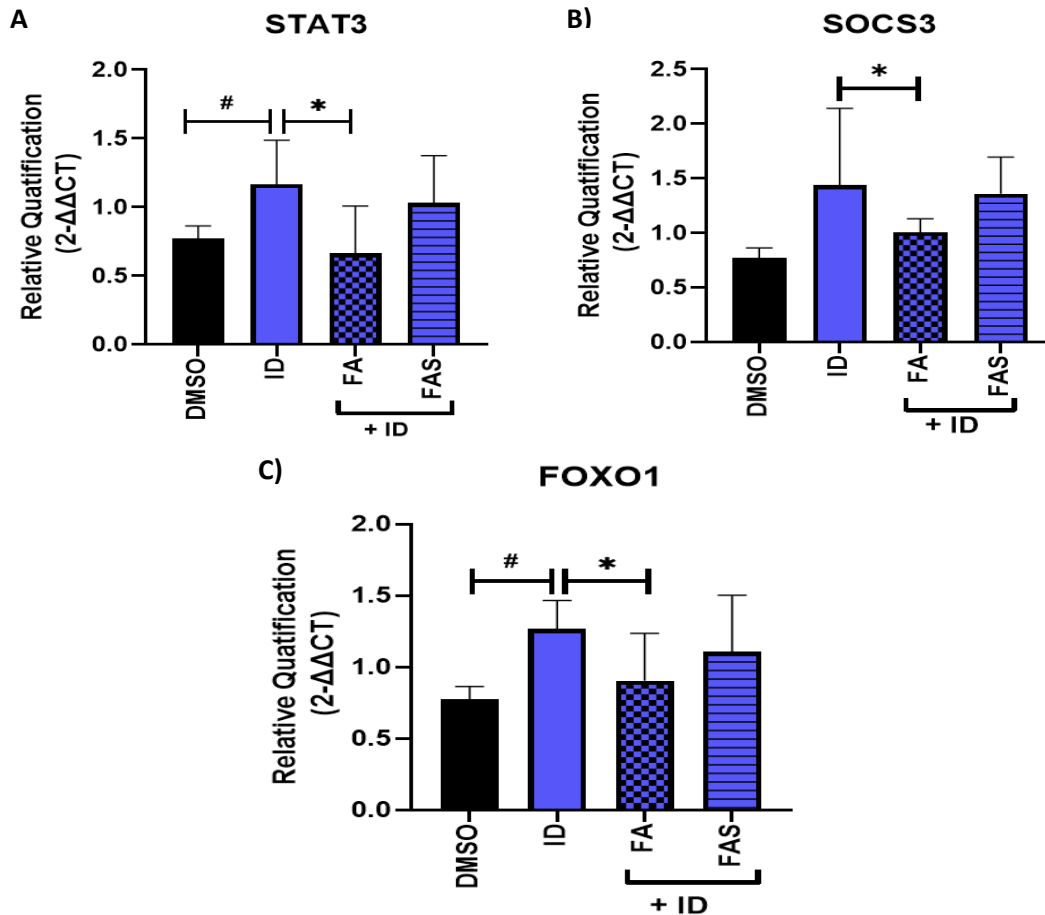


Figure 5.16. mRNA expression of STAT3, SOCS3 and FOXO1 genes in the liver tissues. The mRNA levels of signal transducer and activator of transcription 3 (STAT3) (A), suppressor of cytokine signaling 3 (SOCS3) (B), forkhead box protein O1 (Foxo1) (C) were semi-quantified by RT-PCR analyses in the liver tissues of BALB/c male mice exposed to DMSO (0.1%), Iron dextran (100 mg/kg)(ID), Ferulic acid (20 mg/kg)(FA) or Ferulic Acid 4-O-Sulfate Disodium Salt (20 mg/kg)(FAS) with ID. Administrations of phenolic acids in mice exposed to ID alleviated partially the impaired the mRNA expressions of STAT3, SOCS3 and FOXO1 compared with only ID group. Determination of gene expression by qPCR $2^{-\Delta\Delta CT}$ in the liver and normalized with which housekeeping gene. Data are presented as the mean $\pm$ SEM, $n = 5$ mice per group. # $p < 0.05$ DMSO vs. treatment groups, * $p < 0.05$ ID group only vs. treatments groups. One-way ANOVA, Tukey post-hoc test.

5.3. Discussion

Excessive accumulation of iron-induced ROS or depletion of intermediates with antioxidant capacity alters redox balance and leads to oxidative stress, which is a major cause of various tissue injuries (26). Therefore, the regulation of oxidative stress in tissue is an important strategy to prevent a variety of diseases. Epidemiological, clinical, and nutritional studies strongly support the evidence that dietary phenolic compounds enhance human health by lowering risk and preventing the onset of degenerative diseases including cancers, cardiovascular diseases, and metabolic disorders (27). Ferulic acid, a natural phenolic compound, has been reported to exhibit antioxidant, radical scavenge and metal chelating effects (28). Therefore, the present study was designed and performed to evaluate the ameliorative role of ferulic acid against iron-induced multiple organ dysfunctions and HepG2 cell damage by mediating NRF2 antioxidant pathway. Based on an elaborate study of the concerned signaling pathways, our results demonstrated that ferulic acid provides protection against oxidative stress mediated tissue injuries and cellular dysfunction under excess iron conditions.

The liver is an important organ in the detoxification of compounds. Also, both iron-induced liver damage and antioxidant activities of natural products in the liver are difficult to induce in in vivo models (29). Therefore, in this study HepG2 cells, human hepatoma cells, were chosen to investigate the protective effect of FA against iron-induced oxidative stress. In this in vitro experimental set-up iron-related oxidative stress have been successfully induced in well-differentiated hepatic HepG2 cells. In addition, the present study revealed the free radical scavenging and protective activities of FA in HepG2 cells treated with iron, increasing antioxidant enzyme activities such as SOD and GSH.

The mechanism of antioxidant function of phenolic compounds is complex (14); however, two aspects are of particular interest using phenolic compound FA. Firstly, complete reversal of cytotoxicity, MDA production and GSH loss in response to iron-induced peroxides was observed with treatment of FA or its metabolite FAS. This is mainly based on the inhibition of the formation of ROS since it shows that phenolic acids have ability to permeate cell membranes to reach or adsorption at membrane surface to reach its target due to their lipophilic structure (30). Yang et al. showed that pre-treatment of 5 μ M caffeic acid, lipophilic

phenolic acid, significantly decreased the level of intracellular ROS generation in HepG2 cells treated with 0.3 mM tert-Butyl hydroperoxide (tBHP), oxidative stress inducer (31). Secondly, previous studies have demonstrated that phenolic compounds, beyond their antioxidative activity, can bind transition metal ions such as iron and copper (32). Messner et al. showed increased ROS and oxidative stress in AML-12 mouse hepatocytes following treatment with 50 μ M FAC. In contrast, 50 μ M curcumin treatment decreased iron content, thus leading to lower ROS and MDA levels compared with the control group (33).

With respect to powerful antioxidant properties of dietary phenolics in vitro, they can neutralize free radicals by donating an electron or hydrogen atom to a wide range of reactive oxygen, nitrogen, and chlorine species, including O^{2-} , OH, peroxy radicals RO_2 , hypochlorous acid (HOCl) and peroxyxynitrous acid (ONOOH) (34). Phenolic acids are hydroxyl derivatives of aromatic carboxylic acids which have a single phenolic ring. Hydroxyl groups on the benzoic ring at the ortho-position and/or para-position can lead to elevated antioxidant activity compared to other positions and unsubstituted phenol (35). For instances, in vitro studies showed that phenolic acids inhibited the generation of superoxide anions and hydroxyl radicals in both enzymatic and non-enzymatic systems thus providing protection against lipid peroxidation (36). In vivo study showed that gallic acid treatment decreased the liver iron amount in male C57BL/6J mice with alcoholic liver diseases, thus leading to significantly lower MDA levels compared with healthy mice group. Additionally, liver GSH and SOD levels were reversed by gallic acid treatment in mice with alcoholic liver diseases (37).

In the current study, the effects of excess iron administration against oxidative stress and protective effects of phenolic acids on iron overload were evaluated in in vivo. Liver, spleen, kidney, and pancreas were picked because they have significant relationships with maintaining iron-related oxidative stress process. It has been reported that hepatotoxicity and spleen dysfunction are documented in patients with iron overload (38). This study revealed that significant increases in iron content were reported in mice liver, kidney and spleen tissues following iron treatment (Figure 5.4). The deposition of iron is significantly linked with tissue injuries because it catalyses free-radical reactions. These data are in accordance with previous research reports which suggested a relationship between excess iron accumulation and hepatic diseases such as alcoholic hepatitis, chronic viral hepatitis, and

fibrosis (39). However, supplementations of phenolic compounds, FA, and its metabolite FAS, promoted a decrease in the amount of non-haem iron in tissues, suggesting the interaction on iron metabolism in tissues. Given ferulic acid which has OH group on its aromatic group, essentially binds iron or any bivalent metal; hence its property of iron chelation might reduce the amount of free iron in the body thereby alleviating oxidative stress, both enzymatically and by scavenging free radicals (15). Similarly, excess iron accumulated in the liver after iron dextran treatment in Swiss albino mice whereas tannic acid supplementation decreased liver iron levels in the iron dextran group (40). Additionally, on iron transporter proteins, phenolic acids increased FPN levels, iron exporter, so limiting iron accumulation in liver tissues (Figure 5.14).

Excess oxidative stress was mimicked in iron dextran treatment where high ROS generates imbalance in antioxidant defences, SOD, GSH or MDA. In context, iron dextran supplementation produced a significant elevation in hepatic, splenic and kidney MDA contents as compared with the control group. Administration of FA and FAS significantly reversed iron-induced peroxidative damage in the liver, spleen and kidney as indexed by minor levels of MDA which might be credited to the antioxidant impact of phenolic compounds. Also, it is shown that green tea extracts including phenolic groups (ortho-hydroxy group) reversed lipid peroxidation induced by deposition of excess iron in liver tissue. Thus, this showed that green tea could provide essential antioxidant effects during iron overload, attributing to the presence of phenolic compounds in green tea (41).

GSH in a reduced state is the main intracellular antioxidant, conferring a protective effect against oxidative stress exposure. Additionally, SOD catalyse the dismutation of the superoxide radical (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), providing cellular defence against ROS (42). FA or FAS treatments in iron supplemented mice increased SOD levels in the liver, spleen, and kidney tissues as compared with iron dextran only treatment group. Similarly, sinapic acid treatment, also a phenolic acid, increased the SOD and GSH levels and decreases MDA levels in rat kidney exposed to oxidative stress caused by a high dose of cisplatin (43). Also, FA recovered GSH and SOD levels in murine spleen exposed to high radiation, suggesting ROS scavenging properties of FA (44). All together, these results

showed that the protective effects of phenolic acids might be a consequence of a reduction in oxidative stress damage as well as iron chelation in hepatic, splenic and kidney tissues.

Excessive ROS in the liver can impair the homeostasis of glucose and lipid metabolism and accelerate the progression of hepatic insulin resistance, T2D and NAFLD (45). ROS induce insulin resistance by activating the STAT3-SOCS3 pathway in the liver. Activation of STAT3 by phosphorylation subsequently increases SOCS3 expression through a negative feedback mechanism. SOCS3 then directly disrupts insulin signaling through ubiquitin-mediated degradation of insulin receptor substrate 1 (46). In the present study, FA treatment mitigated the STAT3-SOCS3 signaling in iron overloaded group which can indirectly induce insulin resistance. In parallel, a study by Khodarahmi *et al.* (46) showed that quercetin administration was associated with reduction in mRNA in STAT3 and SOCS3 in the liver of oxidative stressed-model mice. In addition, Foxo1, a key transcription factor, mediates insulin action on gene expression. Under physiological conditions insulin inactivates Foxo1 by phosphorylation-dependent proteins, demonstrating that FA can mitigate iron-induced insulin resistance by activating insulin signalling (47). Research conducted by Dong *et al.* (48) proved that the administration of 500 μ M palmitic acid in HepG2 cells increased the activity of Foxo1 protein in the nucleus whereas 7.5 μ M paeonol treatment, a small molecule with phenolic structure, reversed this effect.

Dietary phenolic compounds are known to exert direct antioxidant effects by ROS scavenging, but also indirect effects through the induction of endogenous protective mechanisms such as NRF2 antioxidant pathway. NRF2, a vital transcription factor, induces the expression of factors that repress oxidative stress, including NQO1, GCLC, GPX4, HO-1, and thus can induce antioxidant activity (49). It is well known that NQO1 and HO-1 are essential for inhibiting redox impairment. HO-1 catalyzes the rate-limiting step of haem degradation to alleviate oxidative damage. NQO1 protects the living organisms against superoxide damages (50). The current study indicated that phenolic acids could induce HO-1 and NQO1 upregulation in the liver of iron-treated mice (Figure 5.12), which is in accordance with a previous study where FA protected against methotrexate-induced kidney injury through the elevation of enzymatic antioxidants; NQO1, HO-1 and Nrf2 (51). Also, caffeic acid activated Nrf2 upregulation under 25 μ M FeSO₄ treatment in rat cortical slices (52). Therefore, phenolic acids can be effective

inducers to activate Nrf2 antioxidant pathway under oxidative stress. Moreover, FA increased the expression of GCLC and GPX4 in iron overloaded liver in this study. GCLC is a rate-limiting enzyme for the synthesis of glutathione while GPX4 is an enzyme that reduces lipid peroxides with glutathione as a co-factor in the process (53). In parallel with these results, 1.2mM FA treatment increased the expression of Nrf2 and GCLC proteins in the auditory murine HEI-OC1 cells (54). Also, ginsenosides, rich in phenolic compounds, diminished ROS production in neuroblastoma SH-SY5Y cells exposed to 6-hydroxydopamine induced oxidative damage by GPX4 expression (55).

In terms of pancreatic tissue injury, iron-related oxidative stress is one of the main causative agents of dysfunction of pancreatic β cells which are sensitive to various types of stress due to their low expression of antioxidant potential (56). It is believed that antioxidant mechanisms can be enhanced by natural compounds in the pancreas, to prevent cell death and insulin deficiency (57). The current study showed increased expression of Nrf2 and its downstream genes after phenolic acid treatments in iron overloaded pancreas. Similar to these results, vitexin treatment, including phenolic compounds, in INS-1 pancreatic B cells exposed to high glucose repaired SOD and GSH levels by activating Nrf2 antioxidant pathway (58). Consequently, it might be thought that phenolic acids increased antioxidant activity in pancreas and so diminishing pancreatic damage and insulin deficiency. Moreover, in the current study, FA treatment increased iron exporter protein, FPN levels in the pancreas, suggesting that phenolic acids can play a role in iron management.

The FA dose used (20 mg/kg) was calculated according to Reagan-Shaw *et al.* (59). The human equivalent dose for the mice is 1.6 mg/kg, which corresponds to around 112 mg of FA consumed daily by adults. The daily recommended FA supplement dose is 100–500 mg, which is also in accordance with the FA dose applied in our study (60).

Ferulic acid is absorbed relatively faster than another phenolic compound from the stomach (61). After absorption, ferulic acid is rapidly formed conjugation product in the liver with glucuronides, sulphate, or sulfoglucuronide. Distribution of ferulic acid in the body after single administration is considerable. Orally administered ferulic acid was distributed almost 4% in gastric mucosa, 10% in blood and liver, kidney, and 53% distributed in other tissues. However,

free FA bioavailability is very low due to the rapid conjugation process in the liver (61). However, it is more bioavailable than other dietary polyphenols so far studied (62).

In general, FA can ameliorate oxidative stress in HepG2 cells and iron treated tissues through Nrf2 signal pathway. A limitation of the study is that FA metabolites were not quantified in the plasma of the experimental mice. In the future study, FA and its metabolites in plasma should be detected by using urine samples. Moreover, a long-term study of FA supplementation is needed to confirm the efficacy and safety. So far, these findings confirm phenolic compounds can trigger antioxidant defence, thus it might be important strategy for treating of oxidative stress-related diseases.

5.4. References

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Chapter 6. General Discussion and Conclusion

6.1. Discussion

Iron is a crucial element in the body, while excess iron accumulation leads to organ dysfunction by the production of ROS (1). In the body, iron is not excreted passively. Therefore, iron tends to accumulate in organs while exogenous iron might be loaded by repeated transfusions, hereditary factors, and other diseased conditions (2). The characteristic consequences of advanced iron accumulation are failure of vital organs such as kidney, heart, liver in addition to endocrine dysfunctions (3). Hereditary hemochromatosis (HH) is the most common genetic disorder by systemic iron overload in Western countries (4) and is reported to be associated with diabetes (5). A positive association between increased body iron stores, estimated as serum ferritin, and incidence of T2D has been consistently reported (5). Similarly, a representation of diabetic cases has also been described among individuals with conditions of acquired iron overload, such as thalassemia major (6). This raises the question whether high levels of body iron are a risk factor for T2D in the general population; this would have implications for the prevention and treatment of T2D.

Insulin resistance is a hallmark and a key factor in the pathogenesis of T2D (7). Although development of overt T2D from insulin-resistant state may take a long time due to an initial increase in islet β cell mass or function, an eventual failure of this compensation leads to impaired β cell functions and body glucose homeostasis. The turning point and the underlying mechanism remain a challenging question (7). Indeed, because of the high demand of insulin, β cells, one of the most metabolically active tissues, consume high amount of oxygen for insulin secretion, which renders β cells to a higher risk of ROS production and oxidative stress. On the other hand, β cells are particularly vulnerable to oxidative stress majorly due to the low level of antioxidant enzymes, which further weakened the ability of β cells in defence against oxidative stress. Overall, metabolic roles of antioxidant mechanism in β cells and diabetes depend on body oxidative status and target functions. Regulatory mechanisms of antioxidants can help prevent iron-induced metabolic complications in humans (8).

Excess iron-related diseases can be treated with iron chelators. There is an emerging role for synthetic iron chelators in the treatment of diseases characterized by oxidative stress; however, their undesirable side effects led to the consideration of natural compounds, which exhibit relatively, minimal degrees of deleterious side effects. Living mechanisms have

evolved with three cellular antioxidant defence systems to cope with ROS related oxidative stress. These include (i) low-molecular-mass antioxidants such as GSH, vitamins C and E; (ii) antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase; and (iii) sequestration and repairing systems. Although all three protective systems are important and interdependent, this study focuses on how natural products have a trigger effect on antioxidant mechanisms during excess iron toxicity (9,10).

The overarching aims of this research were to find protective effects of natural compounds against iron-induced damage in a mouse pancreatic cell line and mouse models. In this study, two specific objectives are:

- 1) to search for evidence of FA against excess iron in cells and mouse tissues
- 2) to investigate the protective pathway of FA against ferroptotic damage in MIN6 cells

In order to determine the beneficial effects of polyphenols against iron associated damage, in vitro and in vivo models were used. In this study, the first set of experiments explored the effects of iron-induced damage and the protective function of polyphenols on cells. MIN6 pancreatic β cells were exposed to Fenton substrate challenge (30 μ M of FeSO_4 and 0.5 mM of H_2O_2) to stimulate iron-induced oxidative damage (11). Also, to mimic iron accumulation in the cells, 50 μ M FAC-20 μ M 8HQ complex was used in vitro (12). Iron dextran, a form of mineral iron can replenish iron stores in a short time, and it is used to induce excess iron in vivo (13). Lastly, erastin provides ferroptotic condition which is a newly discovered type of iron-related cell death (14). To prevent cell and tissue damage against iron, polyphenols were searched in terms of availability and abundance in diet, thus most abundant polyphenols in foods were used to show their beneficial effects in iron associated stresses (15).

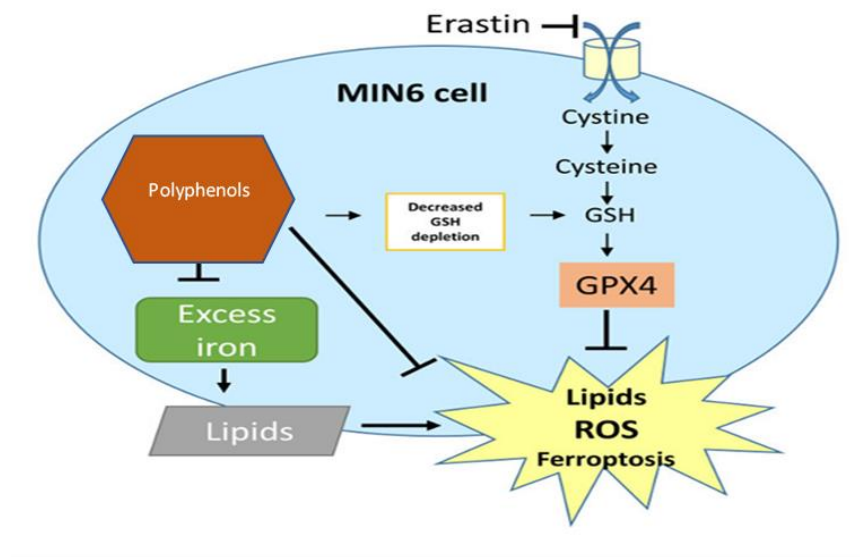


Figure 6.6. Mechanisms of polyphenols under ferroptotic damage

The implication of the first experimental study is the potential benefit of using curcumin and EGCG supplements in the treatment of iron stressor and erastin. In ferroptotic cell death, iron accumulation occurs, thus ROS secondary to excess iron leads to lipid peroxidation which results from a decrease in the activity of GPX4 (16). Curcumin and EGCG decreased the high iron levels in MIN6 cells whereas GSH content increased due to curcumin and EGCG treatments. This led to a reduction in lipid peroxidation in erastin-induced ferroptosis in MIN6 cells. Current treatment of iron overload includes iron chelators, which are hampered by their off-target and side effects. For example, synthetic iron chelators have both a limited capacity to enter cells and are rapidly metabolized, and are risk for hepatic failure, neutropenia, and gastrointestinal disturbances. These therapeutic limitations of currently available iron chelators highlight the need for alternative pharmacological interventions (17). The physicochemical properties of curcumin and EGCG such as hydrophobicity enhances their permeability in the lipid phase of plasma membrane, and this also attributed to their high bioavailability (18).

Glutathione peroxidases (GPXs) are evolutionarily highly conserved enzymes that use GSH as a cofactor to reduce peroxides to their corresponding alcohols, thereby limiting metal-dependent formation of toxic radicals (19). In this study, curcumin and EGCG increased GSH content, suggesting that they can have the ability to neutralise erastin-induced blocked cystine/glutamate antiporter system Xc^- . Yang *et al.* (20) showed that in BJ cell lines (human fibroblast cell lines) inactivation of GPX4 by GSH depletion triggers ferroptosis by

accumulation of the ROS production from lipid peroxidation. In terms of regulation of oxidative stress, curcumin and EGCG reduced the iron levels in cells, thus leading to a decrease in ROS related lipid peroxidation. Similarly, a study by Rainey *et al.* (21) indicated that curcumin showed iron chelating activity, decreasing iron level and decreased lipid peroxidation levels in Huh-7 cells exposed to excess iron. Although curcumin binds iron, there is no significant reduction in iron uptake by cells. Indeed, it is controversial how polyphenols regulate iron metabolism (21).

Increasing studies have indicated that iron related oxidative stress are common features and major factors of pancreatic cell death induced insulin deficiency (22). Therefore, mediators that regulate oxidative stress related molecular mechanisms might be attractive drug targets. ROS accumulation in iron-induced pancreatic cell death is accompanied by a disturbed antioxidant defence system in MIN6 cells (23). Pharmacological evidence has suggested that reducing ROS production or improving antioxidant status is useful for ameliorating cell death or organ dysfunction associated with iron (24,25). Nrf2, known as a cytoprotective transcription factor, has a key role in the basal activity and coordinated induction of targeting gene products, which include antioxidant enzymes (SOD, CAT, GPX4, HO-1, etc), the critical enzymes responsible for glutathione synthesis (GCLC), as well as the major detoxifying enzyme NQO1. These molecules play essential roles in cellular antioxidant defence system (26). Considering the crucial role of oxidative stress related ROS production in the pathogenesis of pancreatic cell damage and insulin deficiency induced by iron, strategies targeting Nrf2 might have a potential for the treatment of excess iron induced diseases for humans. After being activated, Nrf2 translocates to the nucleus to bind with ARE, which is located in the promoter region of genes that encode the antioxidant enzymes. In addition, Keap1 is the known as a repressor protein of Nrf2. In the basal conditions, Keap1 could facilitate the polyubiquitination of the Nrf2 protein, resulting its degradation. Suppressing Keap1 activity could inhibit the degradation of Nrf2 (27). As reported, Nrf2-dependent antioxidant response regulated the protective effect of polyphenols on oxidative-induced insulin deficiency in the diabetic rats (28). In this study, results from both in vivo and in vitro investigations showed that FA and FAS treatments increased the expression levels of Nrf2, as well as the down-streaming signals such as GPX4, HO1, NQO1 and GCLC in MIN6 cells exposed to ferroptotic activity and tissues challenged by iron. Subsequently, these findings

demonstrated that Nrf2 activation by FA and FAS treatments markedly upregulated the antioxidant capacity of MIN6 cells through enhancing the activity of critical antioxidant enzymes such as SOD, and GSH. Meanwhile, treatments of phenolic acids significantly reduced ROS production and MDA levels both in vitro and in vivo. Furthermore, it is believed that polyphenol treatments are capable of inhibiting Keap1, facilitating the subsequent translocation of Nrf2 to the nucleus. These data strongly demonstrated that FA and FAS could protect MIN6 pancreatic cells and tissues from the iron-induced damage by the antioxidative signalling pathway.

The health benefits of phenolic compounds such as FA are attributed to their antioxidant potentials. FA is an extremely abundant, preformed phenolic aromatic chemical found widely in foods (29). In this study, FA, and its metabolite FAS were evaluated on ferroptosis in vitro, and on iron-related oxidative stress in vivo. In the current in vitro investigation, FA, and its metabolite FAS were utilized to reduce the damaging effects of erastin-induced ferroptosis in MIN6 cells. The data indicated that FA could offer marked protection against iron reproductive toxicity by decreasing ROS accumulation and MDA production in erastin exposed to MIN6 murine pancreatic cells, and thus, enhancing in the antioxidant defence system, preventing ferroptotic cell death. To evaluate the molecular changes that underlie the protective effect of FA on erastin-induced ferroptotic cell damage, antioxidant protein expressions were analysed. Erastin-induced ferroptosis markedly reduced the downregulated gene expression of Nrf2 in MIN6 cells. Nrf2 is a redox sensor that regulates the transcription of phase II detoxification enzymes and its downstream signalling pathway (30). The cytoprotective effect of Nrf2 induces the transcription of antioxidant enzymes to eradicate and detoxify the excessive ROS and other electrophilic molecules through phase 1 conjugation reactions, as well as enhancing cellular antioxidant defence capacity (30). The significant effect of FA on Nrf2 activity was studied previously by many researchers (31,32). According to a previous study, FA enhanced Nrf2 expression at levels of transcription and translocation that is from cytoplasm to the nucleus in lead acetate-challenged PC12 cells and upregulated NQO1, GCLC, GCLM, and HO-1 proteins expression through the Nrf2 signalling pathway (32).

In vitro study showed that FA, and its metabolite FAS protected liver, spleen, pancreas, and kidney tissues against iron-induced oxidative damage by activating Nrf2 pathway. The current study showed that oxidative damage might be implicated in tissue injury elicited by iron

dextran. After iron administration, lipid peroxidation levels, oxidative stress marker, are markedly high in liver, spleen, and kidney tissues of BALB/c mice because of the breakdown of antioxidant defence while FA and FAS administration increased tissue antioxidant activities of SOD and GSH under iron dextran treatment. A study showed that impaired antioxidant enzyme activities in hepatic tissues caused an enhanced ROS-induced membrane lipid peroxidation and protein oxidation leading to delayed apoptotic/necrotic cell death in the liver (33). Moreover, Gerin *et al.* (34) stated that the drop in liver SOD, CAT, and GPX activities and elevated MDA levels indicates that formaldehyde impaired the balance between prooxidant and antioxidant balance in favor of prooxidant system. FA treatment improved the antioxidant enzyme activities and decreased MDA levels in the liver of Wistar albino rats (34). This improvement may be due to its free radical scavenging and hepatoprotective effects. FA acts like an antioxidant inducer and neutralizes the ROS and prevents tissue damage. FA is a phenolic compound which has an unsaturated side chain. Its hydroxyl and phenoxyl groups in its phenolic nucleus give their electrons to the free radicals and neutralize them. The electron-donating groups on the benzene ring terminate free radical chain reactions carboxylic acid group with unsaturated C-C bond attracts free radicals and prevents them from destroying cell membrane (35).

T2D represents the final stage of a chronic and progressive syndrome representing a heterogeneous disorder caused by combined effects of insulin resistance and decreased pancreatic β cell functions (36). The liver is the major reservoir of iron in the body and data reported by Houstis *et al.* (37) established that increases in ROS levels can lead to the onset of insulin resistance. Thus, this in vitro study aimed to protect liver and pancreas tissues against oxidative injury by activating Nrf2 pathway. The administration of phenolic acids resulted in increased hepatic and pancreatic Nrf2 and its downstream genes. Furthermore, FPN exports iron in cells and tissues (38). Increased FPN levels were observed after iron administration of FA and FAS in liver and pancreatic tissues of iron dextran and FA&FAS-treated mice, possibly due to increased total iron efflux. Thus, it has been suggested that iron-induced tissue injury can be prevented by phenolic acids due to the upregulation of iron exporter genes.

STAT3/SOCS3 pathway plays a role in insulin action in the liver. STAT3 triggers SOCS3 gene expression under the progression of inflammatory diseases. SOCS3 impairs insulin action by

binding to insulin receptor substrates (IRS1 and IRS2), which leads to ubiquitination and degradation of those receptors (39,40). This study showed that with FA or FAS treatments, iron dextran loaded liver tissues showed reductions in STAT3 and SOCS3 gene expressions; suggesting phenolic acids can ameliorate insulin resistance under oxidative stress. Another study by Khodarahmi *et al.* (41) indicated that the expression of SOCS3 and STAT3 decreased, while the expressions of IRS1-insulin receptors followed an increasing trend after administration of quercetin in the liver of oxidative stress rat models. A similar study conducted in this regard revealed that the application of CCl₄ alters the expression of the STAT3/SOCS3 pathway, leading to hepatotoxicity (42). Rutin, a flavonoid glycoside, protects against CCl₄-induced hepatotoxicity by inverse changes in the expression of the proteins, insulin receptors, STAT3 and SOCS3 (42). In line with this, upregulation of STAT3/SOCS3 pathway can inhibit hepatic insulin signalling via multiple mechanisms, including direct blockade of the insulin receptor, IRS-1/2 whereas phenolic acids can abolish ROS-related stress, thus preventing insulin resistance. Moreover, Foxo1 in various tissues leads to impaired insulin sensitivity and glucose intolerance. In the liver, insulin resistance increases glucose production because of an impaired ability of insulin to suppress the activity of gluconeogenic enzymes (43). In the insulin-resistant murine model, there is increased hepatic glucose production due to a loss in insulin sensitivity; the rates of hepatic gluconeogenesis and glycogenolysis are increased when compared to normal mice because of high level of Foxo1 gene expression. When the same experiment was repeated with Foxo1 gene deleted mice, insulin sensitivity was partially restored, and hepatic glucose production subsequently decreased (44). Briefly, phenolic acids can play a role in both the antioxidant pathway and iron metabolism.

Considering the normal concentrations of all the endogenous antioxidants in cells, the addition of lower concentrations of these polyphenols such as picomolar or nanomolar levels might not be expected to produce any detectable increase in the total cellular antioxidant capacity. The reason might be that polyphenols used in this study are hydrophobic, suggesting polyphenols at lower concentrations have less capacity to cross the cell membrane, thus lower bioavailability (45). Furthermore, polyphenols might be rapidly autooxidised in some cell culture protocols. This process leads to a lowering of polyphenols into the nanomolar concentration range in the cells (46). Therefore, testing of polyphenols at lower concentration

range in cell culture systems in the current study might not represent their effective antioxidant capacities.

The use of mouse MIN6 β -cell line is controversial to investigate treatments for diseases and this is a major limitation of this project. MIN6 cells were used in this study instead of human β -cells which is ideal in vitro model for experimentation of human diseases (17). Consequently, in vivo mouse studies were performed to confirm the data from MIN6 cells. Cell culture and animal models are useful for investigating fundamental mechanisms that are not possible to achieve in humans. The endocrine pancreas consists of aggregates of cells known as the islets of Langerhans, which account for almost 2% of the human adult pancreas. Islets consist of glucagon synthesising α -cells, insulin synthesising β -cells, somatostatin insulin synthesising Δ -cells, and pancreatic polypeptide cells. β -cells comprise approximately 50% of human islets (47) and have an estimated turnover of three months (48). β -cells synthesise and secrete insulin primarily in response to extracellular glucose known as glucose-stimulated insulin secretion (GSIS). However, the use of human cell is not common to analyse GSIS with different sets of experiments due to donor shortages (49). Another problem in the use of in vitro testing of human islets is heterogeneity, resulting from donors of different backgrounds. These limitations suggest the need for of easily available and reliable methods for in vitro research purposes (50). Therefore, several cell lines have been used over the last decades. However, these cell lines present vast differences in the amount of insulin secreted and their insulin-secretory response to glucose and other secretagogues. Of all these β cell lines, rat INS-1 and mouse MIN6 cell lines might serve as a model for islets of Langerhans for GSIS in vitro research (51). MIN6 cells were chosen for experimentation since this cell line enables relatively easy genomic manipulation and allows direct comparison of results to previous studies. Furthermore, pancreatic β cell lines are particularly vulnerable to ROS-related oxidative stress since β -cells produce low levels of ROS-scavenging enzymes. Studies showed that oxidative stress plays a crucial role in impaired INS-1 or MIN6 cell functions (52,53), promoting the inhibition of insulin secretion, and leading to irreversible damage. Furthermore, MIN6 cells are not a pure β -cell line, instead existing as a mixture with other pancreatic endocrine cell types, including cells that express and secrete glucagon (50). Ultimately, in this study, MIN6 cells were used as an in vitro cell model to assess protective effects of polyphenols against iron-induced stress and ferroptosis.

However, there are limitations of the use of MIN6 cell line. They tend to lose the mature β -cell features, including glucose-stimulated insulin secretion (GSIS), in long-term culture. This is probably due to the change in expression of GSIS-responsible genes over time. Additionally, although human β -cell lines and MIN6 cells showed some similarities including phenotypes of insulin responsive genes and susceptibility to oxidative stress, the responses of the human β -cell lines remain unclear. Therefore, conclusions are drawn from data obtained from in vitro experiments which need verification in vivo and human studies.

6.2. Future Directions

This thesis has investigated, discovered, and proposed mechanisms of the action of polyphenols, particularly, curcumin, EGCG, FA and FAS, on iron and antioxidant metabolism in well-established in vivo and in vitro models. The potential effect of phenolic acids in iron metabolism and the maintenance of homeostasis is promising and details of the molecular mechanisms await further studies. The development of natural iron treatments requires many stages to demonstrate the effectiveness and safety of the compound.

In this study Chapter 3 shows that polyphenols, particularly curcumin and EGCG, significantly affects antioxidant enzyme activities in ferroptotic MIN6 cells. Further studies need to investigate the mRNA levels of iron metabolism genes, iron levels in cells and antioxidant enzyme activities to understand and confirm their proposed effects. In addition to this in vitro experiment, potential antioxidant, and iron chelation features of curcumin and EGCG should be investigated with in vivo experiments to determine their safety and effective dose in the possibly animal models before experiencing human trials with iron overload conditions.

Furthermore, the results of Chapter 4 in this thesis show that phenolic acids modulate the expressions of Nrf2 antioxidant protein in MIN6 cells exposed to ferroptosis. In further studies, mRNA levels of the genes in the Nrf2 antioxidant pathway should be determined in ferroptotic MIN6 cells treated with phenolic acids and to correlate with the changes of protein levels.

Regarding in vivo iron overload experiment in mouse model (Chapter 5), it is important to confirm the effect of phenolic acids on plasma iron markers such as serum transferrin saturation (TSAT) and hepcidin levels in tissues. FA metabolites were not quantified in plasma in the current study. Also, it is important to investigate iron deposition by laser ablation and ICP-MS in iron overload conditions after administration of phenolic acids. In future study, FA

and its metabolites in plasma or urine should be detected to confirm their bioavailability. Moreover, a long-term study of FA supplementation is needed to confirm the efficacy and safety in human diets. These findings in the current studies present the evidence that phenolic compounds can trigger antioxidant defence, that might be an important strategy for treating oxidative stress-related diseases. In future, similar studies need to be carried out in humans to establish any possible benefits of natural compounds for those at risk of developing iron-related disorders. Moreover, polyphenols driven nanoparticles can be obtained and used in iron overloaded patients to substitute synthetic therapeutic medications following the determination of safety doses of these polyphenols in humans.

6.3. Conclusion

Pancreatic β MIN6 cells showed ferroptotic activities and FA treatments prevented lipid peroxidation, iron accumulation and decreased GSH levels caused by ferroptosis. The positive effect of FA is associated with their antioxidant and iron chelating properties. Phenolic acids were found to decrease the levels of lipid peroxidation and non-haem iron in the liver, spleen, and kidney of mice with iron overload. Phenolic acids can detoxify iron by subsequently sequestering it within their own structures where it cannot be utilized for the Fenton reaction. FA, and its metabolite FAS have stronger protective effects in liver and pancreas by activating Nrf2 antioxidant pathway. Excess iron treatment activated insulin resistance-related genes, STAT3/SOCS3 and Foxo1 which were suppressed by in the liver of mice administered with phenolic acids. FA and its metabolite FAS might modulate pancreatic cell damage, thus alleviating insulin resistance in iron overloaded subjects. Furthermore, phenolic acids protected the tissues against both oxidative stress and iron-overload by regulating FPN gene expression. This will likely bring phenolic acids as valuable therapeutic options for patients at high risk of chronic iron accumulation.

6.4. References

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Appendix I

Publications and oral communications

Kose T, Vera-Aviles M, Sharp PA, Latunde-Dada GO. Curcumin and (–)-Epigallocatechin-3-Gallate Protect Murine MIN6 Pancreatic Beta-Cells Against Iron Toxicity and Erastin-Induced Ferroptosis. *Pharmaceuticals*. 2019 Mar;12(1):26.

Ameliorative effects of phenolic acids on ferroptosis in MIN6 pancreatic cells. **Department of Nutritional Sciences PhD Student Symposium, King's College London**, London, UK, 2017

Protective effects of flavonoids against ferroptotic cell death. **European Iron Club Meeting**. Zürich, Switzerland, 2018

Protective function of ferulic acid against iron overload in mice. **8th Congress of the International Biolron Society**, Heidelberg, Germany, 2019.

Also, images of Perl's staining liver (Figure 5.5) and spleen (Figure 5.6) were used from Mayra Vera-Aviles' thesis. Mayra and I, we performed the animal experiments together. Thus, those images were used in the two PhD students' dissertations.

Appendix II

Bovine serum albumin (BSA) standards for protein determination

BioRad DC colorimetric assay was used to determine protein concentration of the samples. The kit consisted of reagent A (alkaline copper tartrate solution), reagent B (dilute Folin Reagent), and reagent S. Bovine serum albumin (BSA) standards were diluted as in the following table.

Water (μ l)	BSA (μ l)	Concentration (μ g/ μ l)
105	15	0.25
90	30	0.50
60	60	1.00
30	90	1.50
0	120	2.00