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IN VIVO AND IN VITRO TOXICITY EVALUATION OF IRON FROM RED SEA FISH PRODUCTS AND THE INFLUENCE OF OXIDATIVE STRESS: A POTENTIAL RISK TO HUMAN HEALTH.

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ABSTRACT

Fish species from the Red Sea constitute an excellent food source but are also unsafe to consume because their content of iron (Fe) is greater than the recommended level. We looked into the safety issues connected to the ingestion of iron-rich fish. Fe(II) and Fe(III) were administered to Wistar rats at a dose of approximately 200 mg/kg body weight.Hepatic cell lines (WRL-68, HepG2 and FTO2B) were subjected to iron for cytotoxicity tests.After that, H2O2 was added as a pretreatment to FTO2B cells in order to mimic oxidative stress.The *in vivo* findings demonstrated that only the Fe2+ treatment altered significantly ($P < 0.05$) aminotransferase activity relative to the control and increased antioxidant enzyme activity and malondialdehyde (MDA) concentration. The cell lines did not exhibit toxicity from iron, which is commonly found in Red Sea fish, according to the in vitro results.On the other hand, the detrimental effects of H2O2 on FTO2B cells were enhanced by the addition of Fe(III).Additionally, the data showed that superoxide dismutase gene expression was elevated ($P < 0.05$) following exposure to Fe(III). The data obtained indicates that consuming fish products from the Red Sea may provide a potential risk of toxicity.

Keywords:*Fish; Food quality; Iron; Cytotoxicity; Oxidative stress*

Introduction

Biological activities require iron (Fe), but it is extremely toxic when present in excess. The two main kinds of available Fe are Fe(II) in conditions of reduction within cells and Fe(III) in environments of aerobic metabolism[1]. Iron is a redox-active metal that controls cell development and differentiation as well as oxidation–reduction reactions. It acts as a prosthetic group for several proteins involved in essential cellular functions, including ribonucleotide reductases and DNA polymerases during DNA synthesis, cytochromes in the electron transport chain for cellular respiration, hemoglobin and myoglobin for oxygen transport and energy generation, and numerous enzymatic reactions [2,3]. Many proteins and enzymes that maintain a variety of physiological activities contain iron as an essential

component [4]. Despite the fact that iron is a trace metal present in the diet, anemia affects more than 2 billion people worldwide [5]. Additionally, iron can be toxic in excess due to its capacity to stimulate the generation of hazardous reactive free radicals via the Fenton reaction [6].

The liver is where iron is most commonly stored, and iron can seriously damage other organs [7]. In addition to its role in detoxification, the liver is essential for the metabolism of biological molecules. Most substances that are absorbed pass through the intestinal barrier and enter the liver, where toxic elements can accumulate. When studying the effects of pollution, the liver is the most vital organ to take into consideration [8]. Iron accumulation in hepatic tissues is a secondary effect of frequent blood donation and noticed in patients with long-term liver disorders, such as alcohol-related liver disorders and persistent viral hepatitis [9]. Recent research has revealed that the physiopathology of Fe-induced hepatic damage and cell death may involve oxidative stress, process facilitated by free radicals and reactive oxygen species (ROS) [10].

The Red Sea receives discharge of a significant amount of wastewater from the Jeddah Metropolitan Area daily [11,12]. Each day, this area receives 100,000 m3 of sewage sludge, which lowers the water quality, increases human exposure and ecological and health risks, and causes an overall rise in the mortality of aquatic species [12]. Indeed, heavy metals are regarded as important classes of toxic substances that penetrate aquatic systems by anthropogenic processes and/or atmospheric discharge in addition to organic compounds. Aquatic systems are contaminated with significant quantities of heavy metals, which accumulate and become amplified in sea water, sediment, and the food chain, eventually posing a major threat to human health [8]. In fact, the average amount of Fe discovered in the liver of the fish species *Variola louti* from the contaminated region was 4020.01 µg/g liver tissue [11]. Additionally, according to a recent study, the concentration of iron in the tissues of economically important species of marine fish collected from the coastal region of Jeddah ranged from 81.60 to 188.60 mg/kg dry weight [13].

In a prior study, oxidative stress caused by iron overload produced excessive ROS generation and led to serious liver damage [14]. However, there is insufficient knowledge regarding the use of Fe(II) or Fe(III) as a potential preventive agent against cell injury induced by oxidative stress. Furthermore, it has been recognized that organisms have enzyme-based antioxidant defenses, such as superoxide dismutase (SOD) and catalase (CAT), that are essential for preserving cellular homeostasis by eliminating ROS [15]. Recent research has increasingly focused on the regulation of antioxidant gene expression as a potential indicator of oxidative stress.

Thus, evaluating the effects of Fe(II) and Fe(III) in vivo and in vitro may help to better understand the toxicity or benefits of iron and to evaluate the potential health effects in Red Sea fish after long-term exposure. Therefore, the current investigation was performed to examine the impact of Fe(II) and Fe(III) on cell death and oxidative stress using human and animal liver cell lines and an in vivo rat model. To assess the possible intensification of iron cytotoxicity that occurs via oxidative stress, the toxicity of H2O2 combined with iron to liver cells was investigated to imitate reactive oxygen species generation by the body.

Materials and methods

Solutions

FeSO4 and FeCl3 (50 mM final concentration) were added to sterile H2O to make iron stock solutions. Each day, fresh solutions of Fe(II) and Fe(III) were prepared. The stock solution of H2O2 was 9.79 M.

Animals and experimental design

This study included 30 randomly selected male Wistar rats weighing between 150 and 200 g. The Local Ethics Committee on Animal Experiments of Sfax University approved the experimental procedures (ethics approval number: 1204). The animals were distributed randomly into three groups (n= 8), and they were given a commercially available low-iron diet (5 mg/Kg diet) which are supplied with vitamins, soy, and corn by the Company of Animal Nutrition in Sfax, Tunisia (Table 1). Rats in the Control group (Control) were fed low-iron diet without injection of iron, while rats in the Fe(II)- and Fe(III)-treated groups were given iron intraperitoneally (i.p.) once per week during4 weeks for a total dose of 200 mg/kg body weight. The concentrations were chosen based on the results published by Younis et al.[13], where the iron concentration in the muscle (consumable part) of different commercial and high nutritive value marine fish species from the Jeddah Coast was determined. At the end of the two-week, the animals were anesthetized with pentobarbital (Nembutal, 50 mg/kg), and the abdominal aorta was used to extract blood for hepatic function and oxidative stress parameter analysis. The rats were then sacrificed by exsanguination under anesthesia. Liver samples were collected and fixed in a 10% formaldehyde solution before being stored in 70% ethanol for histological analysis.

Table 1. Composition of rat diet: low-iron diet (LID) contains soya, corn, vitamins, and minerals.

Ingredient	Low-Iron Diet (LID)
Nutritional properties (%)	
Moisture	14
Fibers	3.4
Proteins	22
Lipids	3.5
Ash	6.7
Carbohydrate	50.4
Caloric value ($kcalkg^{-1}$)	2850
Amino acids (%)	
Cysteine	0.38
Methionine	60
Threonine	0.80
Tryptophane	0.30
Mineral mix ($mgkg^{-1}$)	
Iron	5
Manganese	80
Copper	18.75
Zinc	65
Selenium	0.30

Cobalt	0.20
Iode	1.20
Antioxidants and Vitamins ($mgkg^{-1}$)	
Vitamin A	13000
Vitamin H	62.5
Vitamin D3	4375
Antioxidants (BHA-BHT)	125

Liver function

The levels of serum aminotransferases were used as established markers of hepatic injury. Alanine aminotransferase (ALT) was measured using the GPT (ALAT) IFCC mod. kit produced by Human Gesellschaft für Biochemica und DiagnosticambH, Germany, according to Reichling and Kaplan[16]. As stated by Schumann and Klauke[17], aspartate aminotransferase (AST) was determined using the GOT (ASAT) IFCC mod. kit produced by Human Gesellschaft für Biochemica und DiagnosticambH, Germany. Alkaline phosphatase (ALP) was estimated using the DEA Buffer, DGKC kit from Human Gesellschaft für Biochemica und DiagnosticambH, Germany [18].

Oxidative stress parameters

Investigations of superoxide dismutase (SOD), catalase (CAT) and thiobarbituric acid reactive substances (TBARS) can reveal evidence of oxidative stress. These analyses were carried out using a commercially available IBL kit (IBL International, Hamburg, Germany) based on the recommendations provided by the manufacturer.

Liver histology

Fixed tissue fragments were processed, embedded in paraffin for histological examination and cut to a thickness of 5 µm before collection and staining with hematoxylin and eosin.

Cell culture

Hepatic cell lines (rat FTO2B cells, human WRL-68 cells and human HepG2 cells) were used in this study. The stock solutions of FeSO4 (50 mM) and FeCl3 (50 mM) were made in sterile H2O. The different cell lines were cultivated in flasks (75 cm^2) and trypsinization and counting were performed when the cultures were close to confluence. After counting, 24-well plates were used to hold the cells (100,000 cells per well in 1 ml of medium). The medium was taken out after 24 hours and changedwithanother medium containing varying concentrations of iron and H2O2. In the samples

without Fe(II) and Fe(III), equal volumes of media were supplied to cells to serve as controls. Following a further forty-two hours, the medium was taken out and replaced with medium including XTT solution, and the total number of cells was counted. Fe2+ and Fe3+ stock solutions at five concentrations (2500, 1000, 200, 100 and 50 µM) were employed in the cytotoxicity assay. An iron concentration of 100 µM was used in combination with H2O2. These concentrations were chosen based on the results published by Younis et al. [13], in which the iron concentrations in the muscle (consumable part) of five major marine fish from Jeddah Coast, Red Sea were estimated.

Cytotoxicity assay

After 72 hours of culture in iron-containing medium, 500 µl of XTT solution was used to replace the medium. After placing the cells in a CO2 incubator for four hours, the change of XTT product into soluble formazan was determined using a plate reader. To compare cell viability percentages, cells that had not been exposed to iron or H2O2 in the same experiment were employed as controls.

mRNA expression determined by RT–PCR

To gain more knowledge about the expression of genes (CAT and SOD) linked to oxidative stress, rat liver cells (FTO2B) were utilized. In the presence of Fe3+ (100 µM) and H2O2 (880 µM), the cells were cultured to assess gene expression. Four replicates of each condition were used. After 72 hours of exposure, TRIzol reagent was applied to extract the whole RNA from the collected cell samples. In short, 1 ml of TRIzol reagent was used to lyse cells, and then 200 µl of chloroform was added to the sample tubes. The same tubes were then centrifuged at 16,000 × g for 25 minutes at 4 °C. After being transferred to different tubes, the aqueous phase was precipitated with ethanol (70%). In the presence of RNase-free water, the samples were centrifuged at 9000 × g for 1 minute to form RNA pellets. Then, the RNA was stored at -80 °C. The 260/280 nm ratio was used to assess the RNA purity. Using reverse transcriptase, total RNA (2.5 g) was converted to cDNA. mRNA expression was amplified for 35 cycles by PCR using specific primers for SOD and CAT: SOD forward: 5′acaggattaactgaaggcgagcatggg3′, SOD reverse: 5′ccacaccgtcctttccagcagcc3′; CAT forward: 5′cccacgatattaccagatactccaaggc3′, CAT reverse: 5′agtttgccaactggtataagagggtagtcc3′; GAPDH forward: 5′tcatcatctccgccccttccgc3′, and GAPDH reverse: 5′aggcggcatgtcagatccacaacg3′. PCR was used to determine the expression of the genes encoding the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), SOD, and CAT. The SOD, CAT, and GAPDH genes had sizes of 177, 684, and 390 bp, respectively. The SOD, CAT, and GAPDH pixel values were calculated using the Un-Scan-it V6.2 program, after which the agarose gel was digitally photographed. The proportions of CAT and SOD to GAPDH were considered.

Analysis of the results

The data is displayed as the mean ± standard deviation (SD) of a minimum of four independent trials, each of which was performed in duplicate. To perform the statistical comparisons, one-way analysis of variance (ANOVA) proceeded by Fisher's Least Significant Differences test (LSD-test) was employed. $P < 0.05$ was seen as an indicator of statistical significance.

Results

Liver function

At the end of the experiment and following exposure to iron, all of the rats survived with no signs of systemic toxicity. Figure 1 displays the biochemical parameters of liver function. In comparison to the control group, the Fe3+ concentration had no effect on ALT, AST, or ALP activities. In contrast, enzyme activity was significantly augmented inFe2+ group in comparison to the control ($P < 0.01$).

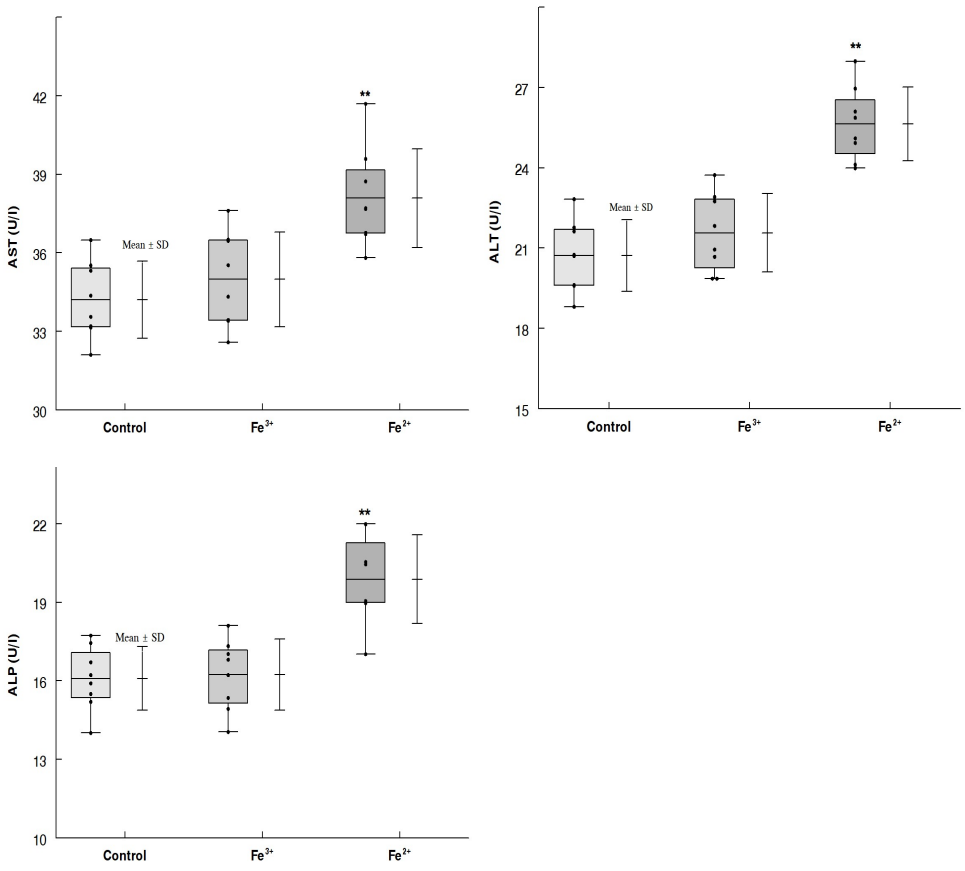

Figure 1. Biochemical markers in male rats following treatments with Fe(II) and Fe(III). Data is shown as mean ± SD. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: Alkaline phosphatase. Using one-way ANOVA, *$P < 0.05$ and **$P < 0.01$ compared to the control.

Effects of Fe2+ and Fe3+ on oxidative stress parameters

Lipid peroxidation was assessed by examining the MDA levels in animal liver tissues. Fe2+ treatment produced a significant increase in MDA content in rat liver compared to the control ($P < 0.01$, Fig. 2). In the Fe3+ group, SOD and CAT activities were normal in comparison to the control. In contrast, the Fe2+ group showed significantly increased levels of SOD and CAT compared with the control groups ($P < 0.05$).

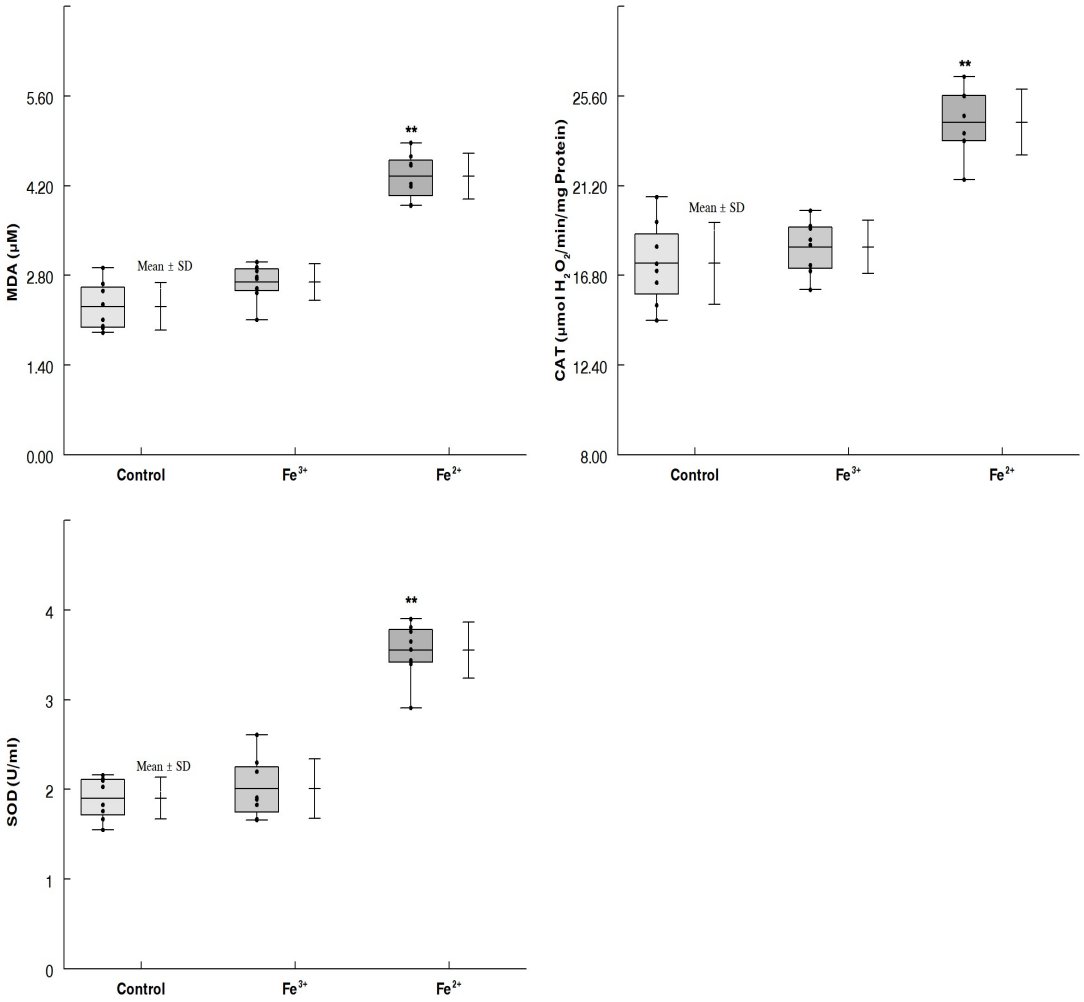

Figure 2. Oxidative stress parameters in male rats following treatments with Fe(II) and Fe(III). Results are presented as mean ± SD. Using one-way ANOVA, *P < 0.05 and **P < 0.01 compared to the control.

Histology

Hematoxylin-eosin staining showed that the hepatic tissues had normal structures in the different rat groups, suggesting that the concentrations of Fe2+ and Fe3+ used in this study did not affect these tissues (Fig. 3).

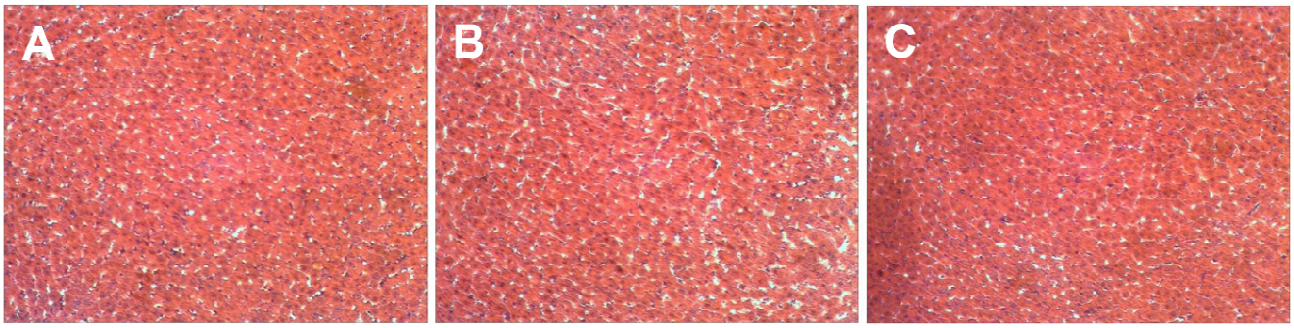

Figure 3. Liver histology from different treatment groups. Hematoxylin-eosin stain showing a normal structure of liver tissues in the different groups. (A) Control group, (B) Fe(II) treated group, (C) Fe(III) treated group (magnification x 200).

Effect of Fe(II) on cellular viability

Figure 4 illustrates the cytotoxicity results measured by XTT assay in the three cell lines used after 72 hours of Fe2+ stimulation. Treatment of FTO2B cells with Fe(II) resulted in a concentration-dependent loss in cell viability. Fe(II) was discovered to be cytotoxic to FTO2B cells at a concentration of 2500 µM. The other cell lines were not damaged by the various Fe(II) concentrations.

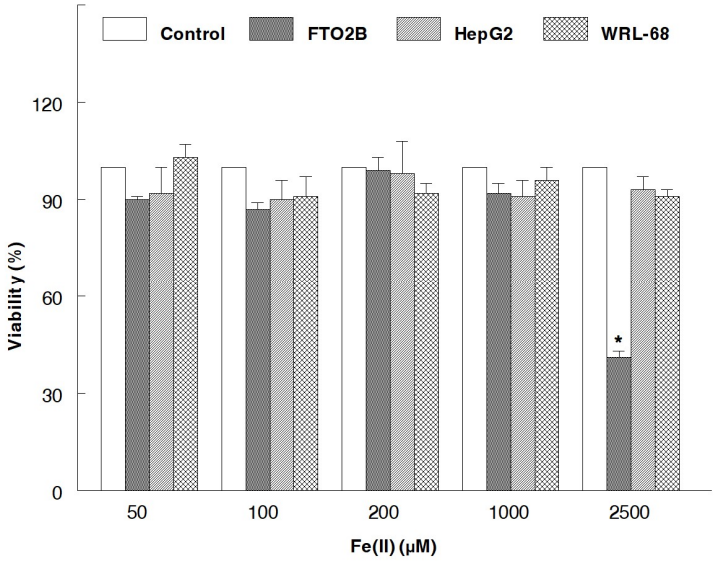

Figure 4. Impact of Fe(II) on hepatic cell lines viability determined after XTT incubation. For 72 hours, the hepatic cell lines were exposed to Fe(II) in rising concentrations. Cell viability of the control was taken to be 100% at the specified concentration. The Results are given as percentage and every value represents mean ± SD. Using one-way ANOVA, *P < 0.05 and **P < 0.01 in comparison to the control. Viability (%): OD/ODcontrol

Effect of Fe(III) on cell viability

The cytotoxicity test showed that Fe(III) had no effect on cellular viability at doses above 2500 µM in the different cell lines (Fig. 5).

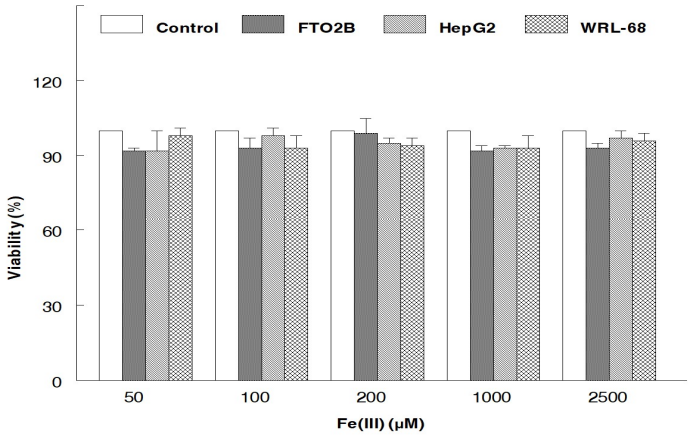

Figure 5. Impact of Fe(III) on hepatic cell lines viability determined after XTT incubation. For 72 hours, the hepatic cell lines were exposed to Fe(III) in rising concentrations. Cell viability of the control was taken to be 100% at the specified concentration. The Results are given as percentage and

every value represents mean ± SD. Using one-way ANOVA, *P < 0.05 and **P < 0.01 compared to the control. Viability (%): OD/ODcontrol

Intracellular Effects of H2O2 and Fe(II) cotreatment

Liver cells were cultured with medium (as a control), H2O2, Fe(II), or H2O2 and Fe(II) together and then charged with XTT. As indicated in Figure 6, H2O2 lowered the mitochondrial reduction of XTT, and cell mortality increased with increasing hydrogen peroxide concentration; 880 µM H2O2 was the first dose to induce significant cell death. A similar result was found in a previous investigation by Charkoudian et al. [19]; Only at 2930 µM H2O2 did cotreatment induce a decrease in cellular viability compared with H2O2 alone.

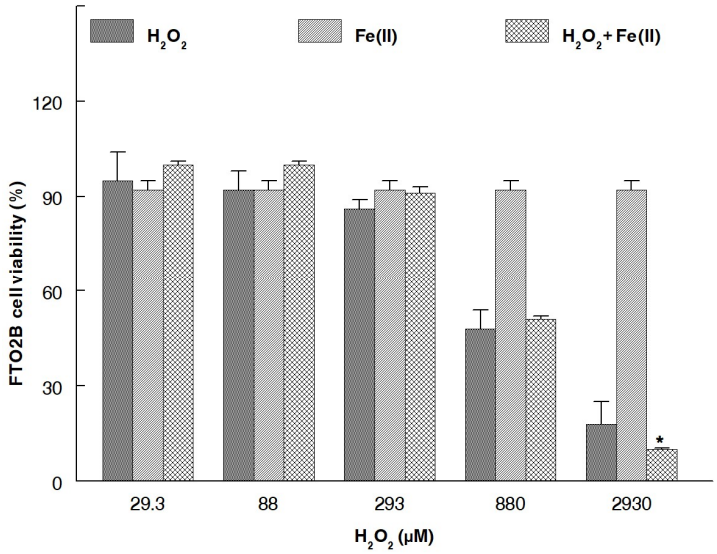

Figure 6. Impact of H2O2 in cotreatment with Fe(II) (100 µM) on hepatic cell lines viability determined after XTT incubation. Cell viability of the control was taken to be 100% at the specified concentration. The Results are given as percentage and every value represents mean ± SD. Using one-way ANOVA, *P < 0.05 and **P < 0.01 compared to the control. Viability (%): OD/ODcontrol

Intracellular Effects of H2O2 and Fe(III) cotreatment

The data from cotreatment with Fe(III) and various H2O2 concentrations are shown in Figure 7. Cellular sensitivity to H2O2 coupled with Fe(III) was shown to differ significantly (P < 0.05) from that to H2O2 alone. The results showed that Fe(III) at nontoxic concentrations could significantly increase H2O2 toxicity, indicating the synergistic effects of H2O2 and Fe(III) in vitro. Similar to Fe(II), cytotoxicity was observed with increased concentrations of H2O2 but was more pronounced with Fe(III).

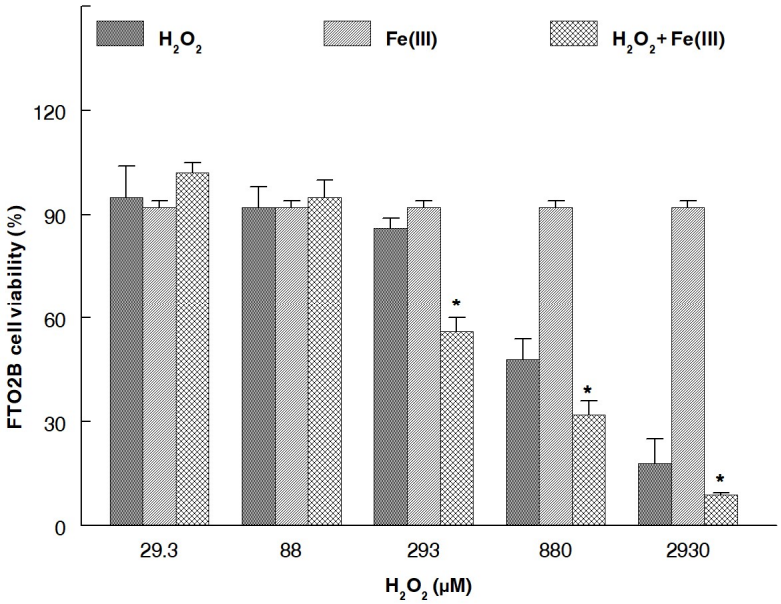

Figure 7. Impact of H2O2 in cotreatment with Fe(III) (100 µM) on the XTT assay's measurement of cell viability. Cell viability of the control was taken to be 100% at the specified concentration. The Results are given as percentage and every value represents mean ± SD. Using one-way ANOVA, *P < 0.05 and **P < 0.01 in comparison to the control. Viability (%): OD/ODcontrol

CAT and SOD mRNA expression

We thought that upregulation of the antioxidant enzymes SOD and CAT would have a protective impact if Fe(II) and Fe(III) exert their cytotoxic effects in part by oxidative stress. To evaluate this hypothesis, FTO2B cells were incubated in the presence of 880 µM H2O2 and 100 µM Fe(III). After 72 h of incubation, Fe(III) produced a significant augmentation in SOD gene expression (P < 0.05) in comparison with the control group (Fig. 8). CAT and SOD mRNA levels were not significantly changed after exposure to 880 µM H2O2.

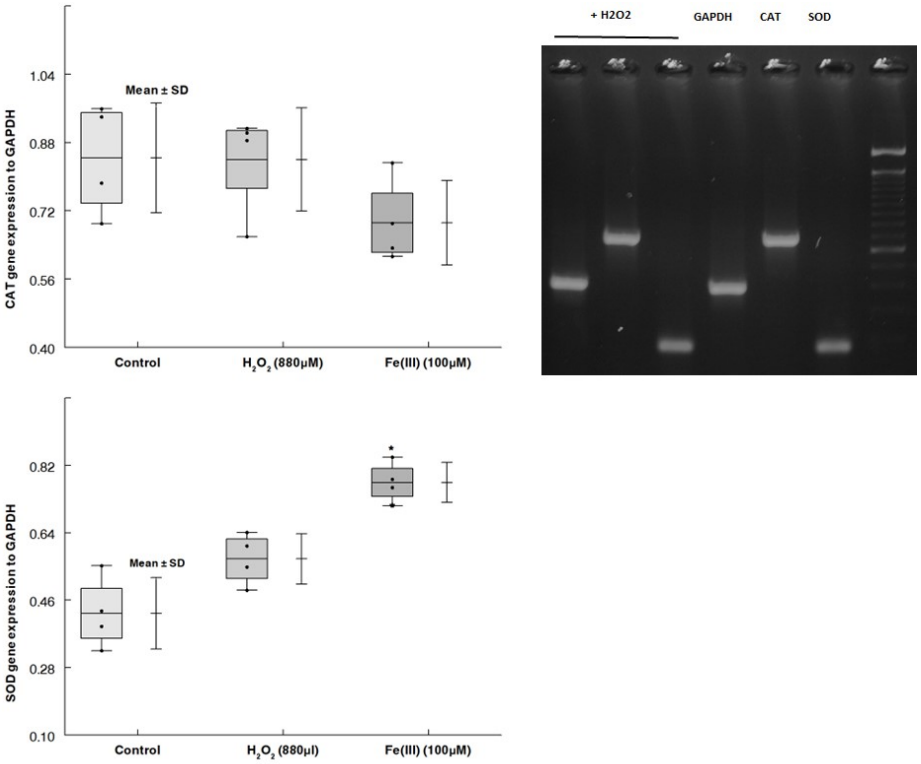

Figure 8. Impact of Fe(III) on SOD and CAT gene expression on FTO2B cells. RT-PCR was used to assess SOD and CAT mRNA expression. We recorded and converted gel images based on pixel value using the software UN-Scan-it V6.2 from Silk Scientific, Orem, UT, US. Every value corresponds to mean ± SD. Using one-way ANOVA, *P < 0.05 and **P < 0.01 in comparison to the control.

Discussion

Iron accumulates in the parenchyma of some organs, such as the heart, pancreas, liver, and endocrine organs, as a characteristic of the pathophysiology of iron excess. Iron overload causes harmful effects in rats, including hepatocellular enlargement, cardiomyopathy, pancreatic atrophy and splenic white pulp atrophy [20]. Iron is a fantastic biocatalyst but is also able to undergo opposite modifications depending on the oxidative environment, making it a potentially dangerous metal [1]. Iron in form of Fe2+ interacts with oxygen to produce free radicals that injure the intracellular contents and lead to cell mortality and has been linked to iron-mediated toxicity. Because iron requires oxygen to promote free radical reactions, excess Fe2+ causes cellular dysfunction [21,22]. Even Fe3+ has often been regarded as noncytotoxic. In this study, intraperitoneal exposure to iron in the form of FeCl3 did not produce toxicologically significant modifications. Only with Fe2+ treatment were the activities of AST, ALP and ALT influenced, indicating the presence of hepatic injury (Fig. 1). Furthermore, microscopic analysis revealed no signs of

liver injury or other inflammatory processes. According to Appel et al. [23], feeding iron in the form of FeSO4 until 11.5 and 11.2 mg/kg body weight/day had no toxicologically significant consequences and did not cause tissue iron overload.

Oxygen free radicals are scavenged by SOD and CAT, which may be indirect indicators of the body's antioxidant capacity [24]. The final product of lipid peroxidation (MDA) is generally used as a bioindicator of lipid peroxidation, is associated with the intensity of the pertinent free radical response and indirectly assesses the level of hepatocyte damage [25]. Our in vivo results revealed that the activities of SOD and CAT were augmented in response to ROS production, as revealed by the increase in MDA content (Fig. 2). In general, the liver is exposed to too much iron more often than other organs because it is the primary and main organ for iron storage [10]. Indeed, our data highlight that Fe2+ at levels normally found in many species of Red Sea fish could pose a risk to human health.

In the in vitro study, we evaluated the oxidant effect of iron in FTO2B cells. In particular, we assessed the involvement of transition metal ions (Fe2+ and Fe3+) in the oxidative stress environment already induced by extracellular H2O2 on cell viability. H2O2 is thought to have the ability to effortlessly penetrate biological membranes and diffuse a great distance from its source since it is an uncharged molecule with a low molecular mass [26]. High levels of H2O2 (more than 50 µM) have often been found to be toxic to many animal, plant and bacterial cells, which is consistent with our results. Indeed, several factors influence how cells die, such as the cell line used, length of exposure to H2O2, iron content, concentration of H2O2 employed, and cell culture medium used [8,27]. In the current work, we found evidence Fe2+ toxicity to only FTO2B cells at high concentrations (2500 µM), and the experimental setup revealed no cell death after Fe(III) treatment. However, the results showed that Fe(III) at a nontoxic concentration (100 µM) significantly increased H2O2 toxicity and markedly augmented cell death above the level caused by H2O2 alone, indicating a synergistic effects between H2O2 and Fe(III). When cotreated with H2O2, there seems to be a cytotoxicity from Fe3+ and no effect or a protection from Fe2+. Chamnongpol et al. [1] reported that Fe3+ can induce cellular toxicity and that Fe3+ mediates its cytotoxicity via a process that is oxygen independent but unlike the process induced by Fe2+. Zhao et al. [28] establish that Fe3+ perform biphasic roles in cultured porcine parthenotes. They showed that redundant Fe3+ conducted to high ROS concentration, lowering of that redundant Fe3+ decreased ROS content, and mitochondrial function is further protected. Although, excessive Fe3+ depletion reduced mitochondrial function, resulting in blastocyst apoptosis. According to Leiter et al. [29], concentrations between 150 and 300 µmol/L of extracellular Fe3+ increased the oxidative stress caused by 700 mmol/L H2O2 when fungi cultured and grown in complex medium. We hypothesized that cotreating cells with H2O2 plus Fe2+ can increase cell injury and consequently cell death. However, to our surprise, the incorporation of Fe2+ did not change the harmful effects of H2O2. This finding is consistent with the outcome provided by Hempel et al. [30], who established that extracellular Fe(II) can defend cells against hydrogen peroxide-induced injury and speculated that extracellular Fe(II) initiates the Fenton reaction on the exterior of the cells where a large amount of hydroxyl radicals (HO•) react with medium components, safeguarding the interior cellular environment from H2O2[30]. According to the authors, the lack of intracellular damage caused by H2O2 and Fe2+ supports the idea that most or all of the

hazardous signal is produced outside of the cell. Additionally, exposure to Fe(III) enhanced SOD mRNA expression. There was plenty of in vitro evidence of cellular damage, including damage from HO•, when these antioxidants are unable to limit extracellular oxidant generation. Furthermore, the apparent Fe(III)-induced expression of antioxidant genes suggests that iron might serve as a physiological signal that mediates the cellular reaction to oxidative stress caused by H2O2.

Conclusions

The in vivo and in vitro observations in the present study indicated that cells must be stressed after consuming moderate amounts of Fe(II) and Fe(III) from Red Sea fish. Thus, information on iron and/or metal concentrations in fish and sea products in general is important to assess the possible exposure of the community to toxic compounds after their consumption. Consequently, determination of the estimated weekly intake of iron by people consuming different species of fish will be significant for human health and safety.

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Conflicts of Interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Author Contributions:

“Conceptualization, Saber Abdelkader. Saïdi. and Othman. Ahmed Alghamdi.; methodology, Saber Abdelkader. Saïdi.; software, Jos. van Pelt; validation, Saber Abdelkader. Saïdi., Othman. A. Alghamdi and Jos. van Pelt.; writing—original draft preparation, Saber Abdelkader. Saïdi.; supervision, Jos. van Pelt. All authors have read and agreed to the published version of the manuscript.”

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author, Saber Abdelkader Saidi, upon reasonable request.

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