Original Article

Effects of Iron Overload on Cardiac Health and the Protective Role of Betaine in Male Rats

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ABSTRACT

Background: Iron is a vital element that takes part in many biological processes; nevertheless, the excess of iron in the body is a serious toxicological risk as it causes oxidative stress and, consequently, damage to the systemic organs. Betaine is a naturally occurring, non-toxic substance that is found in various organisms, and is known to stabilize protein structures and improve the antioxidant defense mechanisms of the body.

Objectives: The aim of the study was to examine the protective role of betaine against cardiac injury and systemic toxicity in case of chronic iron overload using an animal model.

Methods: Thirty-two adult male albino rats were randomly divided into four groups: a negative control group, an iron-overload group (receiving iron sucrose), a treatment group (receiving iron sucrose in combination with oral betaine), and a betaine-only group. Over a 30-day experimental period, systemic iron levels, biomarkers of oxidative stress, and molecular indicators of cardiac injury were evaluated. The cardiac tissues were also subjected to histopathological examination to determine the structural changes.

Results: Iron overload led to a significant increase in iron storage, with increased oxidative stress markers and cardiac injury markers. Histological examination showed that the iron-overload group had severe myocardial damage and inflammation. Conversely, betaine co-administration had a significant mitigating effect, reducing oxidative stress markers and enhancing iron homeostasis. In addition, cardiac tissue analysis showed that there was no evidence of necrosis or structural damage of the cardiac tissue compared to the iron-only group.

Conclusion: Betaine demonstrates a marked protective effect against iron-induced toxicity. Its cardioprotective effects are explained by its capacity to maintain iron homeostasis, decrease oxidative stress, and preserve cellular structural integrity. These results indicate that betaine can be used as a promising therapeutic adjunct in the management of conditions induced by iron overload.

Keywords: Betaine, Iron overload, Cardio protection, Oxidative stress, Iron metabolism

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Introduction

Iron is a basic transition metal and one of the most common elements in the Earth's crust, which combines the essential biological roles with significant toxicological risks (Dev et al., 2017; Muckenthaler et al., 2017). It is an essential cofactor in cellular metabolism, DNA synthesis, and respiration, and plays a key role in oxygen delivery via hemoglobin (Read et al., 2021; Soares et al., 2016). One of the major properties of iron is the possibility to exist in two oxidation states: the ferric (Fe^{3+}) storage form and the ferrous (Fe^{2+}) active form (Ru et al., 2024; Vogt et al., 2021).

Iron metabolism is a very controlled phenomenon since an excess in free iron may lead to systemic toxicity. Non-transferrin-bound iron (NTBI) builds up when the ratio of systemic iron to the binding capacity of proteins, such as transferrin and ferritin exceeds the binding capacity (Arosio et al., 2015; Sukhbaatar et al., 2018). Such surplus iron is a catalyst to the Fenton reaction, which causes the production of deadly hydroxyl radicals that initiate lipid peroxidation and subsequent organ dysfunction in the heart, liver, and kidneys (Gammella et al., 2016; Roemhild et al., 2021; Vogt et al., 2021).

Betaine is a stable, non-toxic, natural compound that is present in different organisms, and was first discovered in the sugar beets (Arumugam et al., 2021; Hoffman et al., 2009). It acts as a compatible osmolyte and a donor of methyl groups and plays a key role in osmotic regulation (Willingham et al., 2020; Zhang et al., 2016). Because of its peculiarities, betaine stabilizes protein structures and increases the antioxidant defenses, in particular, the Nrf2-Keap1-ARE signaling pathway (Bellezza et al., 2018; Craig, 2004; Konstantinova et al., 2008). Betaine can prevent cellular apoptosis by inhibiting caspase-3 expression and thus protect vital organs against oxidative stress (Veskovic et al., 2019).

Aims of the study

The main objective of this study was to examine the protective effect of betaine against the multi-organ injury caused by iron.

The study objectives were to measure *BNP* gene expression and troponin C to assess myocardial stress; assess oxidative stress indicators, particularly malondialdehyde (MDA); and monitor systemic iron parameters, including serum iron, ferritin, and transferrin.

Significance and scope

The study is important because it addresses a gap in the literature concerning the combined cardioprotective effects of betaine under chronic iron-overload conditions (Kumfu et al., 2022; Mancardi et al., 2021). Although the role of betaine in hepatic injury is well documented, its systemic regulatory effects on iron flux and cardiovascular stability represent a new aspect of clinical adjunct therapy (Ashtary-Larky et al., 2022; Yousef et al., 2022). The research was carried out using a 30-day experimental model in adult male albino rats.

Materials and Methods

Experimental animals and ethical approval

Thirty-two adult male albino rats weighing 200–250 g were used in this study. The animals were housed under standard environmental conditions (25 ˚C and 30% humidity) and maintained on a 12-hour light/dark cycle at the Faculty of Veterinary Medicine, University of Kufa. All procedures were approved by the Central Bioethics Committee at the University of Kufa. The rats were randomly divided into four equal groups (n=8 per group) for 30 days:

Negative control group (C-): Received intraperitoneal (I.P.) injections of distilled water.

Positive control group (C+): Received injections of iron sucrose (Jamjoom Pharma, Saudi Arabia) at 80 mg/kg body weight every 7 days (Khristian et al., 2024).

Treatment group (iron + betaine): Received iron sucrose (80 mg/kg every 7 days) and oral betaine (Solarbio, China) at 15 mg/kg through oral gavage.

Betaine group: Received only oral betaine (15 mg/kg) daily (Alipourfard et al., 2023).

Sample collection

Anesthesia was induced in the rats on day 30 by intramuscular injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) (Jirkof et al., 2023). Blood was collected by cardiac puncture, and the samples were centrifuged at 3,000 rpm for 10 minutes to obtain serum. Heart tissues were collected and placed in 10% neutral buffered formalin for histopathological examination.

Laboratory analysis

Gene expression (reverse transcription quantitative polymerase chain reaction [RT-qPCR]): Total RNA was isolated from heart tissue using a TRIzol-based kit (ABM, Canada), and the total RNA concentration was measured using a Quantus™ Fluorometer (Promega, USA). Brain-type natriuretic peptide (Bnp) mRNA levels were measured using a Stratagene MX 3000-P cycler (Germany) and normalized to GAPDH as an internal control. Fold changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ equation, with the positive control group as the calibrator ($2^{-\Delta\Delta Ct}$=1.0000) (Schmittgen et al., 2008).

ELISA Assays: Serum levels of cardiac troponin C, ferritin, transferrin, serum iron, and MDA were determined using specialist rat-specific ELISA kits (Ideal Medica Technologies, China). The samples were read using a Biotek (USA) ELISA reader.

Histopathological study

The fixed heart tissues were dehydrated using varying concentrations of ethanol, cleared with xylene, and embedded in paraffin wax. Sections (4 µm thick) were stained with hematoxylin and eosin (H&E) to assess myocardial health and structural integrity (Bancroft et al., 2013).

A semi-quantitative scoring system was used to assess myocardial damage (0=non-significant occupied lesion - sol, 1=mild pathological changes, 2=moderate pathological changes, 3=severe pathological changes, 4=highly severe pathological changes/tissue damage—point of no return). Five parameters were evaluated: striation loss, interstitial edema, inflammation, hemosiderosis/hemorrhage, and coagulative necrosis (Godbole & Mahajan, 2025).

Statistical analysis

Data were analyzed using GraphPad Prism software, version 10.0. Results are expressed as Mean±SD for eight rats per group (n=8). Differences among the four experimental groups were evaluated using a one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test to identify specific group differences. Statistical significance was pre-defined at $P<0.05$. In the figures, significance levels are indicated as highly significant ($P\leq0.0001$) or non-significant (ns).

Results

Bnp mRNA expression

RT-qPCR analysis showed significant changes in the expression of Bnp mRNA among the experiment groups. The Iron group showed a marked increase in Bnp mRNA expression (1.0000±0) in comparison with the other groups. In contrast, co-treatment with iron and betaine significantly reduced its expression levels to 0.0866±0.06738, a highly significant reduction compared with the iron-only group ($P\leq0.0001$). The fold change of the betaine-only group was 0.0586±0.02245, and no significant difference was found between the iron-plus-betaine group and the betaine-only group (Figure 1).

Oxidative stress and cardiac markers (ELISA): biochemical analyses

MDA: Serum MDA concentrations, used as a marker for lipid peroxidation, were significantly elevated in the Iron group (0.7128±0.03273) compared to the negative control group (0.2787±0.02312) ($P\leq0.0001$). Treatment with iron plus betaine significantly lowered MDA levels to 0.3736±0.06149. The betaine-only group showed an MDA level of 0.2535±0.02136, which was not significantly different from the negative control group (Figure 2).

Cardiac troponin C: A marked increase in serum cardiac troponin C was observed in the iron group (145.4789±4.23515) compared to the negative control group (65.507±6.86153) ($P\leq0.0001$). Administration of betaine in the treatment group significantly attenuated this rise, reducing the concentration to 97.0845±7.94158. No significant difference was detected between the betaine-only group (67.338±5.36363) and the negative control group (Figure 3).

Iron homeostasis parameters

Serum ferritin: The iron group exhibited a drastic increase in serum ferritin levels (73.5027±3.49245) compared to the negative control group (27.4402±4.33678) ($P\leq0.0001$). Co-administration of betaine significantly reduced ferritin levels to (39.1749±1.63789). The betaine-only group remained comparable to the control at 26.9299±2.86514 (Figure 4).

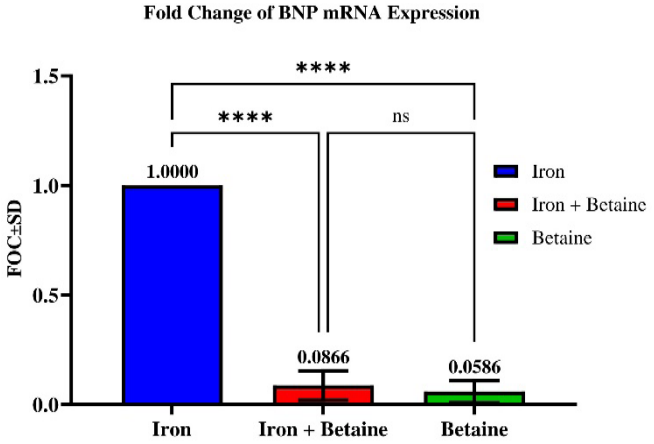


Figure 1. Fold change (FOC) in Bnp mRNA expression

Ns: Not significant.

Note: The bar graph shows the fold change (FOC±SD) in brain-type natriuretic peptide (Bnp) mRNA expression in the experimental groups (n=8) relative to the Iron group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. ****P≤0.0001 indicates a highly significant difference.

Transferrin: Serum transferrin levels were significantly depleted in the iron group (1.4423±0.21956) compared to the negative control group (8.8123±0.20971). However, the iron + betaine group showed a significant recovery of transferrin concentrations to 4.9892±0.39967 (P≤0.0001). The betaine-only group (8.9038±0.21956) showed a non-significant difference compared to the negative control group (Figure 5).

Serum iron: Administration of iron significantly increased serum iron levels in the Iron group (31.4387±1.52911) compared to the negative control group (10.0215±1.46018). Treatment with betaine maintained serum iron at a significantly lower level (12.293±1.16663) than in the iron-only group (P≤0.0001). The betaine-only group had a serum iron level of 9.5712±1.20286 (Figure 6).

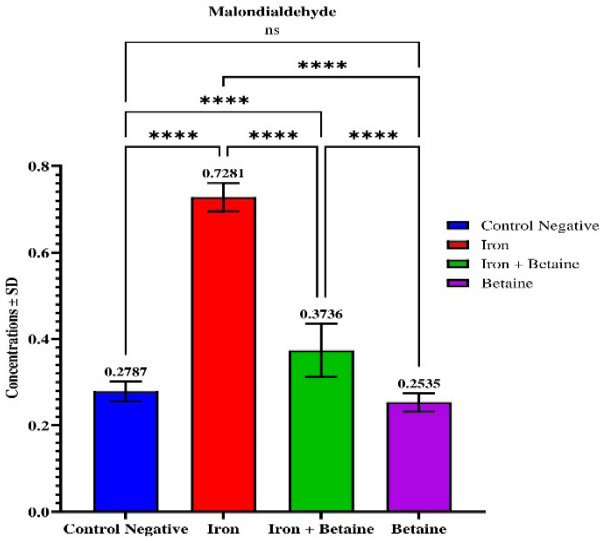


Figure 2. Serum MDA concentrations in the experimental groups

Ns: Not significant.

Note: The bar graph shows serum MDA concentrations (nmol/ml) in adult male albino rats in the control negative, iron, iron + betaine, and betaine groups (n=8). Data are presented as the Mean±SD. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple-comparison test. ****P≤0.0001.

Table 1. Semi-quantitative histopathological lesion scores and tissue damage percentages in different experimental groups.

Groups / Scores	Control	Iron	Iron + Betaine	Betaine
Anucleate cardiac myocyte/losses striated	0	1	1	0
Edema	0	2	1	0
Inflammation	0	3	0	0
Hemosiderosis+ hemorrhage	0	4	0	0
Cardiac muscle fiber coagulative necrosis	0	4	0	0
Total Tissue Damage (%)	0	70-75%	5-10%	0

Note: Scoring was based on a 5-point scale: 0 = No significant occupied lesion (SOL); 1 = Mild pathological changes; 2 = Moderate pathological changes; 3 = Severe pathological changes; 4 = Highly severe pathological changes or tissue damage (Point of no return).

Histopathological findings

Negative control and betaine groups: Microscopic examination of heart sections from the negative control and betaine-only groups revealed normal histological architecture, characterized by healthy texture of cardiac muscle fibers without significant lesions (Figures 7 and 10).

Iron (positive control) group: Heart sections showed extensive coagulative necrosis, loss of striations in cardiomyocytes, and prominent neutrophilic infiltration in the interstitium. Hemorrhagic areas, interstitial edema, and hemosiderin deposition (iron buildup) were also observed (Figure 8).

Iron + betaine group: Sections from this group showed significant attenuation of cardiac injury with betaine treatment. Tissue fragmentation was significantly reduced, and partial restoration of the normal myocyte architecture was observed compared with the positive control group. Slight degenerative changes and residual interstitial separations were also observed (Figure 9).

Histopathological scoring (Table 1) showed also observed. The iron-only group was the most severely affected, with a total tissue damage percentage of 74.375% and grade 4 ("point of no return") coagulative necrosis and hemosiderosis. In contrast, the Iron + betaine group showed significant attenuation of injury across most parameters. Lesions were significantly reduced, and only a few parameters remained slightly affected (grade 1) compared with the positive control group. Tissue damage was very slight (6.875%).

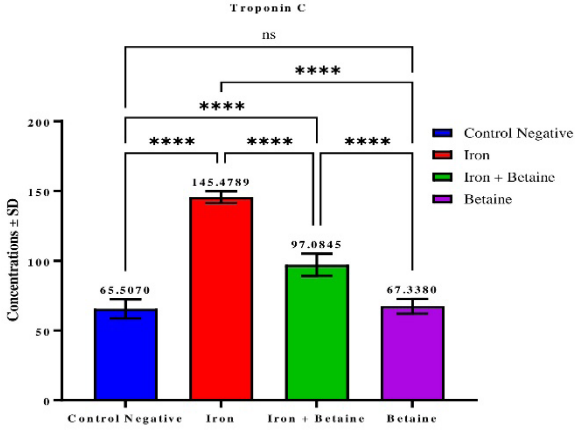


Figure 3. Serum troponin C concentrations in the experimental albino rat groups

Ns: Not significant.

Note: The bar graph shows serum cardiac troponin-C concentrations (pg/mL) in the four experimental group (n=8). Values are expressed as the Mean±SD and were determined using an ELISA. Significant differences are indicated by ****P≤0.0001.

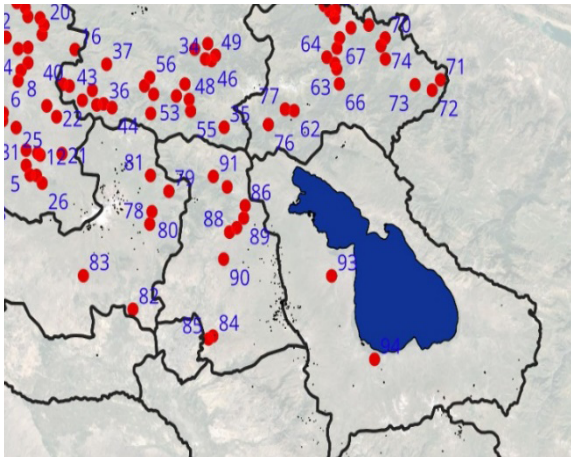


Figure 4. Serum ferritin concentrations in the experimental albino rat groups

Note: The bar graph shows serum ferritin concentrations (ng/mL) in the control negative, iron, iron + betaine, and betaine groups (n=8). Data were quantified using a sandwich ELISA and are expressed as the Mean±SD. Statistical significance is denoted by ****P≤0.0001.

Discussion

The findings of this research show that long-term iron exposure causes systemic toxicity characterized by severe oxidative stress, mechanical and structural strain, and substantial damage to heart tissue. Betaine, a multi-target protective agent, was able to counter these pathological alterations by modulating iron homeostasis and improving antioxidant defenses.

Myocardial stress and injury markers

The iron-overloaded group was used as the calibrator, and a value of 1.000 was assigned to the highest level of myocardial molecular stress. The decrease in Bnp expression to 0.0866-fold in the treatment group represents a significant quantitative decrease in the molecular signaling associated with heart failure. This finding suggests that betaine could reduce genetic markers of cardiac wall stretch by more than 90% compared to the untreated iron-overloaded state, bringing the molecular environment closer to that of the negative control.

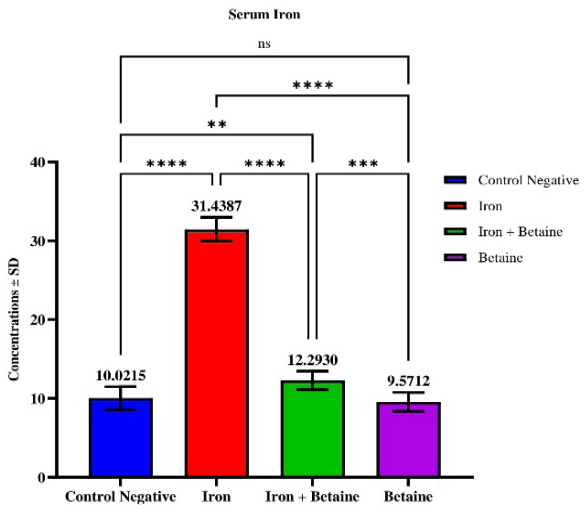


Figure 5. Serum iron concentrations in the experimental albino rat groups

Ns: Not significant.

Note: The bar graph shows serum iron concentrations (µmol/L) in the experimental groups (n=8). Data are presented as the Mean±SD and were quantified using an ELISA. Statistical comparisons were conducted using one-way ANOVA followed by Tukey's multiple-comparison test. P≤0.0001.

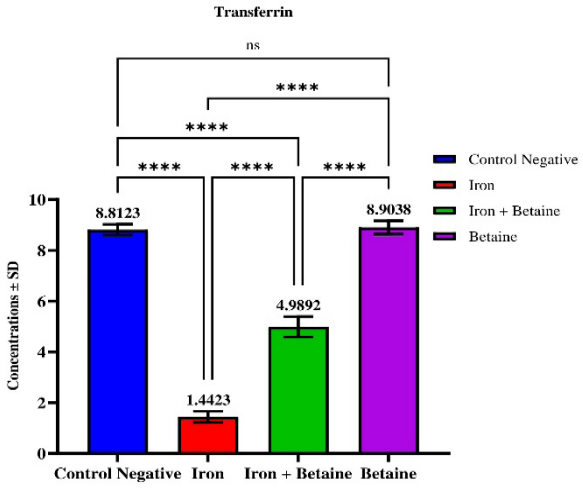


Figure 6. Serum transferrin concentration in the experimental groups of albino rats

Ns: Not significant.

Note: The bar graph shows serum transferrin levels (g/L) in the experimental groups (n=8). Data are reported as the Mean±SD and were measured using a commercial ELISA kit. Significant differences are denoted by ****P≤0.0001.

The significant increases in Bnp mRNA expression (Figure 1) and serum cardiac troponin C (Figure 3) in the iron-overloaded group are key indicators of ventricular dysfunction and cardiac wall stress. Bnp is a sensitive biomarker of iron-overload cardiomyopathy (IOC) and can indicate left ventricular impairment before clinical symptoms appear (Murphy et al., 2010). Excessive iron accumulation places excessive mechanical stress on the myocardial walls, prompting the compensatory release of Bnp. It also increases cardiomyocyte membrane permeability, leading to the leakage of troponin C into the serum (Das et al., 2015).

Betaine treatment significantly lowered these indicators, suggesting improved hemodynamic stability. This cardioprotective effect may be mediated by betaine's

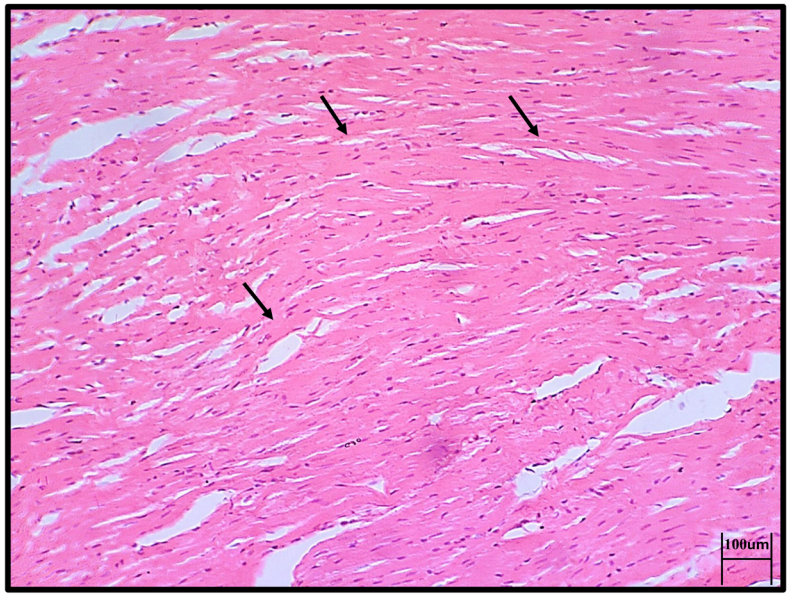


Figure 7. Histopathological section of the heart in the control negative group (H&E; magnification: ×10)

Note: The photomicrograph shows the normal histological appearance of cardiac muscle fibres (black arrows), without any significant space-occupying lesions (SOL).

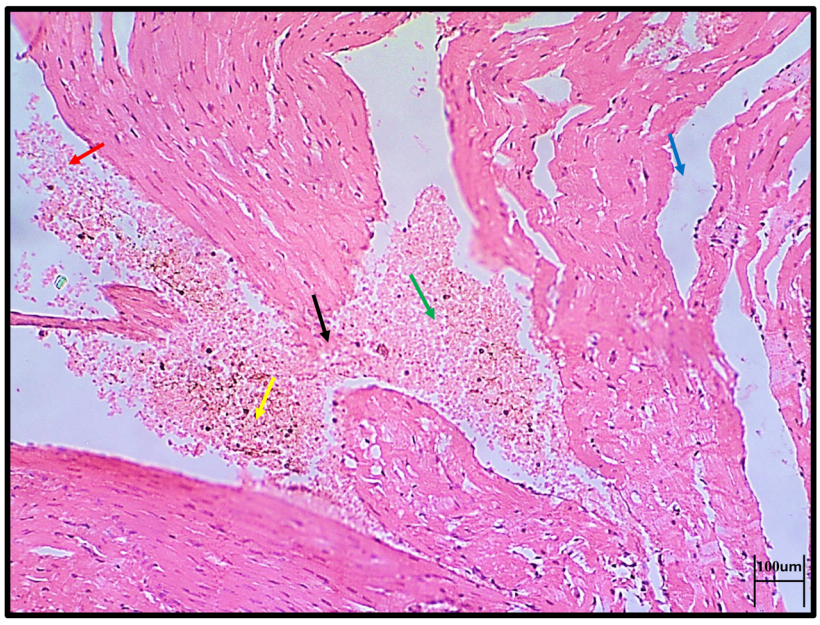


Figure 8. Histopathological section of the heart in the control positive group. The section shows myocardial tissue with extensive coagulative necrosis (black arrow), loss of striations (blue arrow), and prominent neutrophilic infiltration in the interstitium (red arrow). Hemorrhage and interstitial edema are also visible (green arrow), as is hemosiderin deposition (yellow arrow), an indicator of iron-overload injury. Stained with H&E; magnification: ×10.

ability to stabilize energy levels, preserve mitochondrial ATP levels, and maintain cellular membrane integrity (Alirezaei et al., 2017; Ganesan et al., 2010).

Oxidative stress and lipid peroxidation

Pronounced lipid peroxidation is evidenced by the significant elevation of serum MDA levels in the iron-overloaded group (Figure 2). This chemical shift is

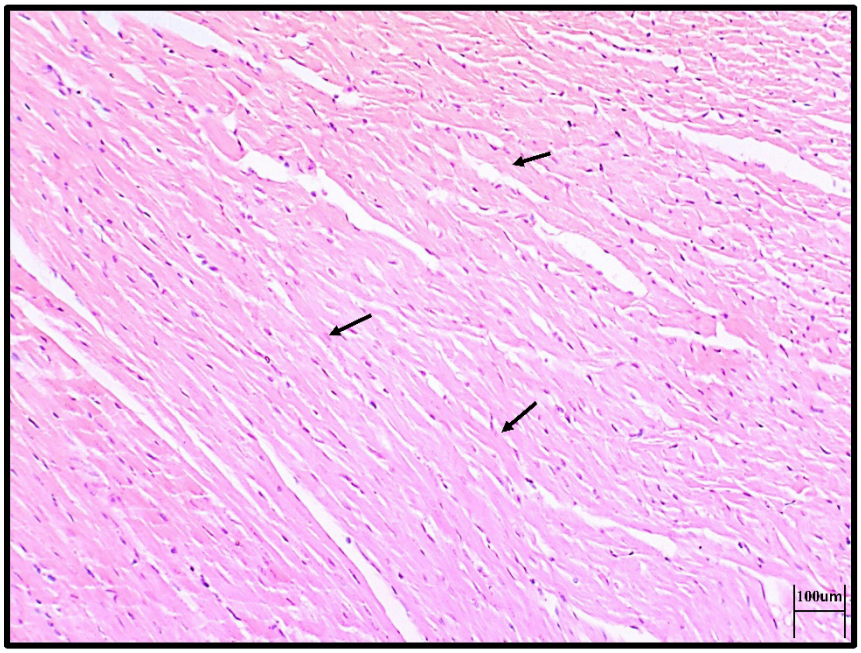


Figure 9. Histopathological section of the heart in the Iron + Betaine treatment group (H&E; magnification: ×10)

Note: The section shows a significant decrease in tissue fragmentation and restoration of normal cardiac muscle fiber architecture (black arrows) compared with the positive control group. A small amount of residual interstitial separation remained, but there were no major space-occupying lesions (SOL).

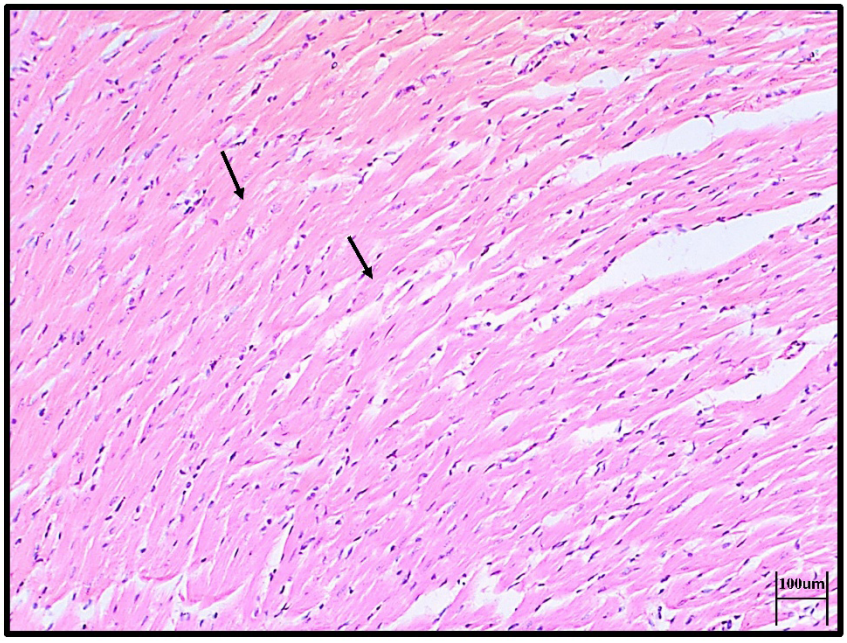


Figure 10. Histopathological section of the heart in the betaine-only group (H&E; magnification: ×10)

Note: The photomicrograph shows normal cardiac muscle fiber architecture, similar to that in the negative control group, with no significant space-occupying lesions (SOL; black arrows).

catalyzed by the Fenton reaction, in which excess free iron promotes the formation of hydroxyl radicals that vigorously attack cellular membranes (Bartfay et al., 2000). Betaine's ability to inhibit MDA generation demonstrates a strong anti-peroxidative effect, consistent with its effects on enhancing glutathione (GSH) activity and activating the Nrf2-Keap1-ARE signal transduction pathway (Erman et al., 2004; Li et al., 2019; Na et al., 2019).

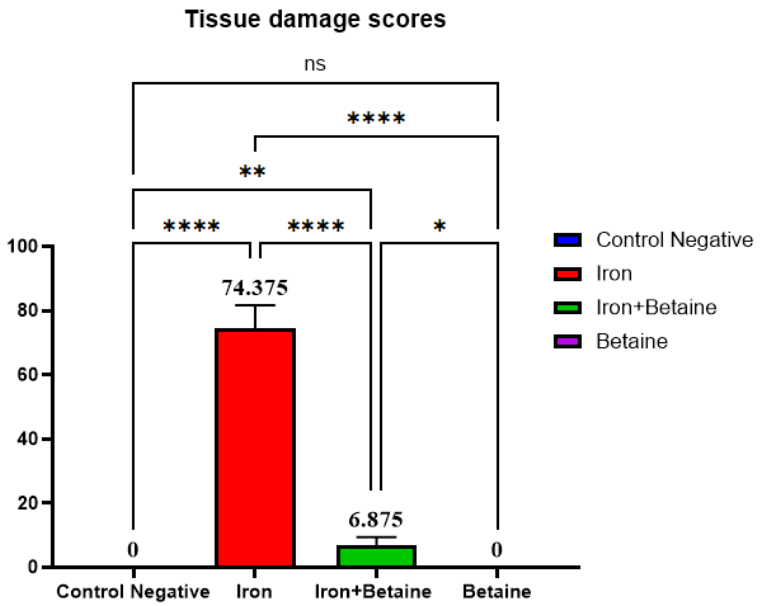


Figure 11. Semi-quantitative evaluation of myocardial injury

Note: The bar graph shows lesion severity (0–4) by group (n=8). Iron overload caused severe damage (74.375%), and treatment with betaine significantly reduced myocardial injury (6.875%). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test.

Systemic iron homeostasis

Systemic iron overload is demonstrated by the marked increases in serum ferritin (Figure 4) and serum iron (Figure 6). Ferritin is also a major marker of iron-store saturation (Paterek et al., 2018; Vogt et al., 2021). The reported decrease in serum transferrin (Figure 5) is probably a compensatory mechanism by which the liver restricts further iron transport to tissues because of excessive iron stores (Vogt et al., 2021). Betaine partially restored transferrin levels and reduced iron stores, highlighting its importance as a key regulator of iron flux and storage (Arumugam et al., 2021; Yousef et al., 2022).

Histopathological findings

Coagulative necrosis and the presence of hemosiderin (Figure 8), both characteristic of iron-mediated tissue injury and ferroptosis, provide histopathological evidence of such damage (Fang et al., 2020; Yan et al., 2019). Conversely, the negative control (Figure 7) and betaine-only (Figure 10) groups exhibited normal myocardial architecture, indicating that betaine is a nontoxic compound with no effect on cardiac histology under normal conditions. The partial restoration of myocyte structural integrity in the treatment group (Figure 9) suggests that betaine has the potential to act as a chemical chaperone that preserves the structural integrity of contractile proteins by protecting against oxidative tissue injury in iron-loaded conditions (Das et al., 2015; Toya et al., 2020).

The semi-quantitative scoring system adds analytical depth by corroborating the severe myocardial injury caused by iron and the marked protective effect of betaine (Table 1; Figure 11). The iron-overloaded group reached the "point of no return" for myocardial injury at Grade 4, with 74.375% damage (Figure 11). This resulted directly from the breakdown of myocardial architecture caused by iron-mediated ferroptosis and excessive oxidative stress, which led to the compensatory elevation of Bnp and the leakage of troponin C (Das et al., 2015, Fang et al., 2020, Murphy et al., 2010).

The significant decrease in tissue damage, from 74.375% to minimal residual damage of 6.875% after betaine administration, indicates that betaine is a potent multitarget protective agent (Table 1; Figure 11). This structural preservation is consistent with the molecular suppression of Bnp expression by more than 90% (0.0866-fold). Betaine has been found to function as a chemical chaperone that stabilizes the integrity of contractile proteins, as well as an energy stabilizer that helps maintain mitochondrial ATP levels (Alirezaei et al., 2017; Das et al., 2015; Ganesan et al., 2010; Toya et al., 2020).

Betaine is able to regulate systemic iron homeostasis, thereby preventing the heart from progressing to irreversible pathological stages (Bellezza et al., 2018; Gammella et al., 2016; Roemhild et al., 2021). The present results indicate that betaine not only attenuates myocardial injury, but also substantially protects against cardiac structural deterioration, which is usually caused by chronic iron-mediated oxidative stress.

Study limitations

Although the present study produced significant findings, it has some limitations that should be noted. First, although H&E staining provided clear evidence of myocardial injury and hemosiderin deposition, the absence of specific iron staining, such as Prussian blue, limits the definitive localization and quantification of focal iron distribution. Finally, the current 30-day experimental model focuses on the subacute-to-chronic phase of iron overload. Long-term observations and assessments of different betaine dose responses are suggested to further investigate its long-term clinical potential.

Conclusion

The present study shows that systemic toxicity is associated with iron overload and is characterized by increased oxidative vulnerability and structural heart damage. Betaine administration was an effective multitarget protective agent against these pathological changes. Betaine protects the heart by decreasing biomarkers of myocardial stress (Bnp and troponin C), inhibiting lipid peroxidation via the Nrf2 pathway, and regulating systemic iron homeostasis. In general, betaine is a potential non-toxic natural protective agent that can protect vital organs against iron toxicity and may serve as an effective remedial therapy for iron-loading illnesses and related cardiomyopathy.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Central Bioethics Committee at the University of Kufa, Kufa, Iraq (Code: No. T/2744).

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Authors' contributions

Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, visualization, project administration, and writing the original draft: Haider Mawash; Review, editing, and final approval: All authors; Supervision: Saif Al-Aqbi.

Conflict of interest

The authors declared no conflict of interest.

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